TOCOPHEROL BIOSYNTHESIS RELATED GENES AND USES THEREOF

This application claims the benefit of U.S. Provisional Application No. 60/400,689 filed August 5, 2002, which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention is in the field of plant genetics and biochemistry. More specifically, the invention relates to genes associated with the tocopherol biosynthesis pathway, and uses of such genes.

Tocopherols are an important component of mammalian diets. Epidemiological evidence indicates that tocopherol supplementation can result in decreased risk for cardiovascular disease and cancer, can aid in immune function, and is associated with prevention or retardation of a number of degenerative disease processes in humans (Traber and Sies, *Annu. Rev. Nutr.*, 16:321-347, 1996). Tocopherol functions, in part, by stabilizing the lipid bilayer of biological membranes (Skrypin and Kagan, *Biochim. Biophys. Acta.*, 815:209, 1995); Kagan, *N.Y. Acad. Sci.*, p 121, 1989); Gomez-Fernandez *et al.*, *Ann. N.Y. Acad. Sci.*, p 109, 1989), reducing polyunsaturated fatty acid (PUFA) free radicals generated by lipid oxidation (Fukuzawa *et al.*, *Lipids*, 17:511-513, 1982), and scavenging oxygen free radicals, lipid peroxy radicals and singlet oxygen species (Diplock *et al.*, *Ann. N Y Acad. Sci.*, 570:72, 1989); Fryer, *Plant Cell Environ.*, 15(4):381-392, 1992).

The compound α-tocopherol, which is often referred to as vitamin E, belongs to a class of lipid-soluble antioxidants that includes α, β, γ, and δ-tocopherols and α, β, γ, and δ-tocotrienols. α, β, γ, and δ-tocopherols and α, β, γ, and δ-tocotrienols are sometimes referred to collectively as "vitamin E". Vitamin E is more appropriately defined chemically as the beneficial activity for animals and humans which can be e.g., determined in the rat fetal absorption and hemolysis assays (Chow, *Vitamin E*, In: Handbook of Vitamins ISBN:0-8247-0428-2). α-Tocopherol has the highest vitamin E activity, in part because it is readily absorbed and retained by the body (Traber and Sies, *Annu. Rev. Nutr.*, 16:321-347, 1996). However, other tocopherols and tocotrienols such as β, γ, δ-tocopherols and tocotrienols also have significant health and nutritional benefits.

Tocopherols are synthesized only by plants and certain other photosynthetic organisms, including cyanobacteria. As a result, mammalian dietary tocopherols are obtained almost exclusively from these sources. Plant tissues vary considerably in total tocopherol content and tocopherol composition, with α-tocopherol the predominant tocopherol species

found in green, photosynthetic plant tissues. Leaf tissue can contain from 10-50 μg of total tocopherols per gram fresh weight, but the edible parts of most of the world's major staple crops (*e.g.*, rice, corn, wheat, potato) produce low to extremely low levels of total tocopherols, of which only a small percentage is α-tocopherol (Hess, Vitamin E, α-tocopherol, *In* Antioxidants in Higher Plants, R. Alscher and J. Hess, Eds., CRC Press, Boca Raton. pp. 111-134, 1993). Oil seed crops generally contain much higher levels of total tocopherols, but α-tocopherol is present only as a minor component in most oilseeds (Taylor and Barnes, *Chemy Ind.,* 722-726, Oct., 1981).

The recommended daily dietary intake of 15-30 mg of vitamin E is quite difficult to achieve from the average American diet. For example, it would take over 750 grams of spinach leaves, in which α-tocopherol comprises 60% of total tocopherols, or 200-400 grams of soybean oil to satisfy this recommended daily vitamin E intake. While it is possible to augment the diet with supplements, most of these supplements contain primarily synthetic vitamin E, having eight stereoisomers, whereas natural vitamin E is predominantly composed of only a single isomer. Furthermore, supplements tend to be relatively expensive, and the general population is disinclined to take vitamin supplements on a regular basis. Therefore, there is a need in the art for compositions and methods that either increase the total tocopherol production or increase the relative percentage of α-tocopherol produced by plants.

In addition to the health benefits of tocopherols, increased tocopherol levels in crops have been associated with enhanced stability and extended shelf life of plant products (Peterson, *Cereal-Chem.*, 72(1):21-24, 1995); Ball, *Fat-soluble vitamin assays in food analysis. A comprehensive review,* London, Elsevier Science Publishers Ltd. (1988). Further, tocopherol supplementation of swine, beef, and poultry feeds has been shown to significantly increase meat quality and extend the shelf life of post-processed meat products by retarding post-processing lipid oxidation, which contributes to the undesirable flavor components (Sante and Lacourt, *J. Sci. Food Agric.*, 65(4):503-507, 1994); Buckley *et al.*, *J. of Animal Science*, 73:3122-3130, 1995).

There is a need in the art for nucleic acid molecules encoding enzymes involved in tocopherol biosysnthesis, as well as related enzymes and antibodies for the enhancement or alteration of tocopherol production in plants. There is a further need for transgenic organisms expressing those nucleic acid molecules involved in tocopherol biosynthesis, which are capable of nutritionally enhancing food and feed sources.

SUMMARY OF THE INVENTION

The present invention includes and provides substantially purified nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, encoding a phytol kinase polypeptide, or polypeptide having phytol kinase activity, encoding a yeast phytol kinase polypeptide, or a yeast polypeptide having phytol kinase activity, encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, encoding a phytol kinase polypeptide or polypeptide having phytol kinase activity comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 69-78.

The present invention includes and provides plant specific phytol kinase motifs (SEQ ID NOs: 74, 77, and 78) and cyanobacterial specific motifs (SEQ ID NOs: 71-73) and nucleotides encoding the same.

The present invention includes and provides a DNA construct comprising a heterologous promoter that functions in plants operably linked to a nucleic acid molecule encoding a phytol kinase polypeptide, or a polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 6, 20-68, and 79 or comprising an amino acid sequence having at least about 70%, 80%, 90%, 95%, or 99% identity to such amino acid sequences.

The present invention includes and provides a transformed plant and progeny thereof comprising an introduced nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of: (1) SEQ ID NOs: 1, 5, and 17 and sequences having at least about 70, 80, 90, 95 or 99% identity to such sequences; (2) an introduced nucleic acid molecule encoding a polypeptide comprising an amino acid sequence having at least about 70, 80, 90, 95 or 99% identity to a sequence selected from the group consisting of SEQ ID NOs: 2, 6, 20-68, and 79; (3) an introduced nucleic acid molecule encoding a phytol kinase polypeptide, or a polypeptide having phytol kinase activity; (4) an introduced nucleic acid molecule encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity; (5) an introduced nucleic acid molecule encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity; (6) an introduced nucleic acid molecule encoding a phytol kinase polypeptide, or a polypeptide having phytol kinase activity comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 69-78; (7) an introduced nucleic acid molecule encoding a plant

phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity comprising an amino acid selected from the group consisting of SEQ ID NOs: 2, 6, and 37-68; (8) an introduced nucleic acid molecule encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-27, and 29-34; (9) an introduced nucleic acid molecule encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid selected from the group consisting of SEQ ID NOs: 74, 77, and 78; (10) an introduced nucleic acid molecule encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid selected from the group consisting of SEQ ID NOs: 74, 77, and 78, wherein said polypeptide is not derived from *Allium porrum, Brassica napus, Gossypium, Glycine max, Oryza sativa, Sorghum bicolor, Triticum aestivum,* and *Zea mays;* (11) an introduced nucleic acid molecule encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 74, 77, and 78 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 75 and 76; (12) an introduced nucleic acid molecule encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 74, 77, and 78 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 75 and 76, wherein said polypeptide is not derived from *Allium porrum, Brassica napus, Gossypium, Glycine max, Oryza sativa, Sorghum bicolor, Triticum aestivum,* and *Zea mays*; (13) an introduced nucleic acid molecule encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 71, 72, and 73; (14) an introduced nucleic acid molecule encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 71, 72, and 73, wherein said polypeptide is not derived from *Synechocystis, Aquifex aeolicus, Chlorobium tepidum, Chloroflexus aurantiacus, Nostoc punctiforme, Prochlorococcus marinus, Rickettsia conorii, Rickettsia prowazekii, Rickettsia sibirica, Synechoccus, Thermosynechoccus elongatus, Trichodesmium erythraeum* and *Saccharomyces cerevisiae;* (15) an introduced nucleic acid molecule encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising one or more amino acid

sequences selected from the group consisting of SEQ ID NOs: 71, 72, and 73 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 69 and 70; (16) an introduced nucleic acid molecule encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising one or more amino acid sequences selected from the group consisting of SEQ ID NOs: 71, 72, and 73 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 69 and 70, wherein said polypeptide is not derived from *Synechocystis, Aquifex aeolicus, Chlorobium tepidum, Chloroflexus aurantiacus, Nostoc punctiforme, Prochlorococcus marinus, Rickettsia conorii, Rickettsia prowazekii, Rickettsia sibirica, Synechoccus, Thermosynechoccus elongatus, Trichodesmium erythraeum* and *Saccharomyces cerevisiae;* (17) an introduced nucleic acid molecule encoding a yeast phytol kinase polypeptide, or a yeast polypeptide having phytol kinase activity; (18) an introduced nucleic acid molecule encoding a yeast phytol kinase polypeptide, or a yeast polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 35 and 36; and, optionally, further comprising one or more additional introduced nucleic acid molecule(s) encoding enzyme(s) or coding region(s) of enzyme(s) of the tocopherol biosynthetic pathway, for example, MT1, tMT2, GMT, tyrA (*e.g.*, SEQ ID NO: 16), HPT (*e.g.*, SEQ ID NO: 15), tocopherol cyclase, dxs, dxr, GGPPS, HPPD (SEQ ID NO: 14), AANT1, IDI, chlorophyllase (SEQ ID NOs: 18 and 19), and GGH (SEQ ID NO: 13), as described in Table 1.

The present invention includes and provides methods for increasing at least one of tocopherol and tocotrienol levels in a plant relative to a plant of similar genetic background but lacking the introduced nucleic acid molecule(s).

In one embodiment, the transformed plant produces seed having at least one of increased tocopherol and tocotrienol levels relative to a seed having a similar genetic background but lacking the introduced nucleic acid molecule(s).

In one embodiment, the transformed plant is selected from the group consisting of alfalfa, *Arabidopsis thaliana*, barley, *Brassica campestris*, oilseed rape, broccoli, cabbage, citrus, canola, cotton, garlic, oat, *Allium*, flax, an ornamental plant, peanut, pepper, potato, rapeseed, rice, rye, sorghum, strawberry, sugarcane, sugarbeet, tomato, wheat, poplar, pine, fir, eucalyptus, apple, lettuce, lentils, grape, banana, tea, turf grasses, sunflower, soybean, chick peas, corn, *Phaseolus*, crambe, mustard, castor bean, sesame, cottonseed, linseed, safflower, and oil palm.

The present invention includes and provides a method for reducing tocopherol levels in a plant comprising: (a) transforming a plant cell with a nucleic acid molecule, the nucleic acid molecule having a promoter region which functions in plant cells to cause the production of an mRNA molecule, wherein the promoter region is linked to an inhibitory nucleic acid molecule complementary to at least a portion of SEQ ID NOs: 1, 5, and 17 or a sequence having at least about 70, 80, 90, 95, or 99% identity to such sequence; and (b) growing the transformed plant cell into a fertile plant; and (c) selecting for a plant with reduced tocopherol levels.

The present invention includes and provides a method of increasing the production of tocotrienols in a plant comprising (a) transforming a plant cell with a nucleic acid construct which causes the down regulation of SEQ ID NOs: 1, 5, or 17 or a nucleic acid sequence having at least about 70, 80., 90, 95, or 99% identity to such sequence; (b) growing the transformed plant cell into a fertile plant; and (c) selecting for a plant with increased tocotrienol levels.

The present invention includes and provides a method for screening for agents that alter tocopherol levels in a plant, comprising: (a) providing a plant lacking a polypeptide comprising the polypeptide sequence of SEQ ID NOs: 2, 6, 20-68, and 79; (b) exposing the plant to a test agent; and (c) assaying tocopherol levels in the plant.

In another preferred embodiment, expression or over-expression of a phytol kinase of the present invention in a transformed plant may provide tolerance to a variety of stresses.

DESCRIPTION OF THE NUCLEIC ACID AND AMINO ACID SEQUENCES

SEQ ID NO: 1 represents an LTT1 nucleic acid sequence from *Arabidopsis thaliana.*

SEQ ID NO: 2 represents a polypeptide sequence encoded by an LTT1 nucleic acid sequence from *Arabidopsis thaliana.*

SEQ ID NO: 3 represents a mutant LTT1 nucleic acid sequence from *Arabidopsis thaliana.*

SEQ ID NO: 4 represents a polypeptide sequence encoded by a mutant LTT1 nucleic acid sequence from *Arabidopsis thaliana.*

SEQ ID NO: 5 represents LTT1-r, a nucleic acid sequence related to LTT1 from *Arabidopsis thaliana.*

SEQ ID NO: 6 represents a polypeptide sequence encoded by an LTT1-r nucleic acid sequence from *Arabidopsis thaliana.*

SEQ ID NO: 7 represents nucleic acid sequence DNA primer 404.

SEQ ID NO: 8 represents nucleic acid sequence DNA primer 405.

SEQ ID NO: 9 represents nucleic acid sequence DNA primer 1652-e-1-f.

SEQ ID NO: 10 represents nucleic acid sequence DNA primer 1652-i-2-r.

SEQ ID NO: 11 represents nucleic acid sequence DNA primer 1652-i-3-f.

SEQ ID NO: 12 represents nucleic acid sequence DNA primer 1652-e-4-r.

SEQ ID NO: 13 represents a nucleic acid sequence of an *Arabidopsis thaliana* GGH.

SEQ ID NO: 14 represents a nucleic acid sequence of an *Arabidopsis thaliana* HPPD.

SEQ ID NO: 15 represents a nucleic acid sequence of an *Arabidopsis* HPT.

SEQ ID NO: 16 represents a nucleic acid sequence of an *Erwinia herbicola* TyrA.

SEQ ID NO: 17 represents a nucleic acid sequence of a *Synechocystis* LTT1.

SEQ ID NO: 18 represents a nucleic acid sequence of an *Arabidopsis thaliana* Chlorophyllase 1.

SEQ ID NO: 19 represents a nucleic acid sequence of an *Arabidopsis thaliana* Chlorophyllase 2.

SEQ ID NO: 20 represents a phytol kinase polypeptide sequence from *Aquifex aeolicus* VF5.

SEQ ID NO: 21 represents a phytol kinase polypeptide sequence from *Chlorobium tepidum* TLS 1.

SEQ ID NO: 22 represents a phytol kinase polypeptide sequence from *Chlorobium tepidum* TLS 2.

SEQ ID NO: 23 represents a phytol kinase polypeptide sequence from *Chloroflexus aurantiacus.*

SEQ ID NO: 24 represents a phytol kinase polypeptide sequence from *Nostoc punctiforme* 1.

SEQ ID NO: 25 represents a phytol kinase polypeptide sequence from *Nostoc punctiforme* 2.

SEQ ID NO: 26 represents a phytol kinase polypeptide sequence from *Nostoc punctiforme* 3.

SEQ ID NO: 27 represents a phytol kinase polypeptide sequence from *Prochlorococcus marinus* 1.

SEQ ID NO: 28 represents a dolichol kinase polypeptide sequence from *Prochlorococcus marinus* 2.

SEQ ID NO: 29 represents a phytol kinase polypeptide sequence from *Rickettsia conorii.*

SEQ ID NO: 30 represents a phytol kinase polypeptide sequence from *Rickettsia prowazekii*.

SEQ ID NO: 31 represents a phytol kinase polypeptide sequence from *Rickettsia sibirica*.

SEQ ID NO: 32 represents a phytol kinase polypeptide sequence from *Synechococcus sp*.

SEQ ID NO: 33 represents a phytol kinase polypeptide sequence from *Thermosynechococcus elongatus* BP-1.

SEQ ID NO: 34 represents a phytol kinase polypeptide sequence from *Trichodesmium erythraeum* IMS101.

SEQ ID NO: 35 represents a dolichol kinase polypeptide sequence from *Saccharomyces cerevisiae*.

SEQ ID NO: 36 represents a Hsd1 polypeptide sequence from *Saccharomyces cerevisiae*.

SEQ ID NO: 37 represents a phytol kinase polypeptide sequence from *Allium porrum*.

SEQ ID NO: 38 represents a phytol kinase polypeptide sequence from *Brassica napus* 1.

SEQ ID NO: 39 represents a phytol kinase polypeptide sequence from *Brassica napus* 2.

SEQ ID NO: 40 represents a phytol kinase polypeptide sequence from *Gossypium hirsutum* 1.

SEQ ID NO: 41 represents a phytol kinase polypeptide sequence from *Gossypium hirsutum* 2.

SEQ ID NO: 42 represents a phytol kinase polypeptide sequence from *Glycine max* 1.

SEQ ID NO: 43 represents a phytol kinase polypeptide sequence from *Glycine max* 2.

SEQ ID NO: 44 represents a phytol kinase polypeptide sequence from *Glycine max* 3.

SEQ ID NO: 45 represents a phytol kinase polypeptide sequence from *Glycine max* 4.

SEQ ID NO: 46 represents a phytol kinase polypeptide sequence from *Oryza sativa* 1.

SEQ ID NO: 47 represents a phytol kinase polypeptide sequence from *Oryza sativa* 2.

SEQ ID NO: 48 represents a phytol kinase polypeptide sequence from *Oryza sativa* 3.

SEQ ID NO: 49 represents a phytol kinase polypeptide sequence from *Oryza sativa* 4.

SEQ ID NO: 50 represents a phytol kinase polypeptide sequence from *Oryza sativa* 5.

SEQ ID NO: 51 represents a phytol kinase polypeptide sequence from *Oryza sativa* 6.

SEQ ID NO: 52 represents a phytol kinase polypeptide sequence from *Oryza sativa* 7.

SEQ ID NO: 53 represents a phytol kinase polypeptide sequence from *Sorghum bicolor* 1.

SEQ ID NO: 54 represents a phytol kinase polypeptide sequence from *Sorghum bicolor* 2.

SEQ ID NO: 55 represents a phytol kinase polypeptide sequence from *Sorghum bicolor* 3.

SEQ ID NO: 56 represents a phytol kinase polypeptide sequence from *Triticum aestivum 1.*

SEQ ID NO: 57 represents a phytol kinase polypeptide sequence from *Triticum aestivum 2.*

SEQ ID NO: 58 represents a phytol kinase polypeptide sequence from *Triticum aestivum 3.*

SEQ ID NO: 59 represents a phytol kinase polypeptide sequence from *Zea mays* 1.

SEQ ID NO: 60 represents a phytol kinase polypeptide sequence from *Zea mays* 2.

SEQ ID NO: 61 represents a phytol kinase polypeptide sequence from *Zea mays* 3.

SEQ ID NO: 62 represents a phytol kinase polypeptide sequence from *Zea mays* 4.

SEQ ID NO: 63 represents a phytol kinase polypeptide sequence from *Zea mays* 5.

SEQ ID NO: 64 represents a phytol kinase polypeptide sequence from *Zea mays* 6.

SEQ ID NO: 65 represents a phytol kinase polypeptide sequence from *Zea mays* 7.

SEQ ID NO: 66 represents a phytol kinase polypeptide sequence from *Zea mays* 8.

SEQ ID NO: 67 represents a phytol kinase polypeptide sequence from *Zea mays* 9.

SEQ ID NO: 68 represents a phytol kinase polypeptide sequence from *Sorghum bicolor* 4.

SEQ ID NO: 69 represents a cyanobacterial motif 1.

SEQ ID NO: 70 represents a cyanobacterial motif 2.

SEQ ID NO: 71 represents a cyanobacterial motif 3.

SEQ ID NO: 72 represents a cyanobacterial motif 4.

SEQ ID NO: 73 represents a cyanobacterial motif 5.

SEQ ID NO: 74 represents a plant motif 1.

SEQ ID NO: 75 represents a plant motif 2.

SEQ ID NO: 76 represents a plant motif 3.

SEQ ID NO: 77 represents a plant motif 4.

SEQ ID NO: 78 represents a plant motif 5.

SEQ ID NO: 79 represents represents a phytol kinase polypeptide sequence from *Synechocystis.*

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates a schematic representation of the tocopherol biosynthesis pathway.

Figure 2 illustrates the plasmid map of pMON36525.

Figure 3 illustrates the plasmid map of pMON69914.

Figure 4 illustrates the plasmid map of pMON77670.

Figure 5 illustrates the plasmid map of pMON81019.

Figure 6 illustrates the plasmid map of pMON77637.

Figure 7 illustrates the plasmid map of pMON78621.

Figure 8 illustrates the plasmid map of pMON81063.

Figure 9 illustrates the plasmid map of pMON69969

Figure 10 illustrates a phylogenetic tree.

Figure 11 illustrates cyanobacterial motif 1.

Figure 12 illustrates cyanobacterial motif 2.

Figure 13 illustrates cyanobacterial motif 3.

Figure 14 illustrates cyanobacterial motif 4.

Figure 15 illustrates cyanobacterial motif 5.

Figure 16 illustrates plant motif 1.

Figure 17 illustrates plant motif 2.

Figure 18 illustrates plant motif 3.

Figure 19 illustrates plant motif 4.

Figure 20 illustrates plant motif 5.

DETAILED DESCRIPTION

The present invention provides a number of agents, for example, nucleic acid molecules and polypeptides associated with the synthesis of tocopherol, and provides uses of such agents.

Tocopherol Biosynthesis

The plastids of higher plants exhibit interconnected biochemical pathways leading to secondary metabolites including tocopherols. The tocopherol biosynthetic pathway in higher plants involves condensation of homogentisic acid and phytylpyrophosphate to form 2-methylphytylplastoquinol (Fiedler *et al.*, *Planta*, 155:511-515 (1982); Soll *et al.*, *Arch. Biochem. Biophys.*, 204:544-550 (1980); Marshall *et al.*, *Phytochem.*, 24:1705-1711 (1985). This plant tocopherol pathway can be divided into four parts: 1) synthesis of homogentisic acid (HGA), which contributes to the aromatic ring of tocopherol; 2) synthesis of phytylpyrophosphate, which contributes to the side chain of tocopherol; 3) joining of HGA and phytylpyrophosphate via a prenyltransferase followed by a methylation reaction, a subsequent cyclization; 4) and another S-adenosyl methionine dependent methylation of an

aromatic ring, which affects the relative abundance of each of the tocopherol species. *See* Figure 1.

Various genes and their encoded proteins that are involved in tocopherol biosynthesis are listed in the table below.

Table 1. Tocopherol biosynthetic coding regions and enzymes

Coding region or Enzyme Abbreviation	Enzyme name
tyrA	Mono or bifunctional prephenate dehydrogenase
HPT	Homogentisate prenyl transferase
DXS	1-Deoxyxylulose-5-phosphate synthase
DXR	1-Deoxyxylulose-5-phosphate reductoisomerase
GGPPS	Geranylgeranyl pyrophosphate synthase
HPPD	p-Hydroxyphenylpyruvate dioxygenase
AANT1	Adenylate transporter
IDI	Isopentenyl diphosphate isomerase
MT1	Bacterial 2-methylphytylplastoquinol methyltransferase
tMT2	Plant 2-methylphytylplastoquinol methyltransferase
GGH	Geranylgeranyl diphosphate reductase
slr1737	Tocopherol cyclase
GMT	Gamma Methyl Transferase
LTT1	Phytol kinase
Chl1 and Chl2	Chlorophyllase 1 and 2

As used herein, homogentisate prenyl transferase (HPT), phytylprenyl transferase (PPT), slr1736, and ATPT2, each refer to proteins or genes encoding proteins that have the same enzymatic activity.

As used herein, a phytol kinase is an enzyme that phosphorylates free phytol and/or phosphorylates phytol monophosphate. "Having phytol kinase activity" means that the enzyme phosphorylates free phytol and/or phosphorylates phytol monophosphate.

Synthesis of Homogentisic Acid

Homogentisic acid is the common precursor to both tocopherols and plastoquinones. In at least some bacteria, the synthesis of homogentisic acid is reported to occur via the conversion of chorismate to prephenate and then to p-hydroxyphenylpyruvate via a bifunctional prephenate dehydrogenase. Examples of bifunctional bacterial prephenate dehydrogenase enzymes include the proteins encoded by the *tyrA* genes of *Erwinia herbicola* and *Escherichia coli.* The *tyrA* gene product catalyzes the production of prephenate from chorismate, as well as the subsequent dehydrogenation of prephenate to form p-hydroxyphenylpyruvate (p-HPP), the immediate precursor to homogentisic acid. p-HPP is

then converted to homogentisic acid by p-hydroxyphenylpyruvate dioxygenase (HPPD). In contrast, plants are believed to lack prephenate dehydrogenase activity, and it is generally believed that the synthesis of homogentisic acid from chorismate occurs via the synthesis and conversion of the intermediates arogenate, tyrosine, and p-hydroxyphenylpyruvate. Since pathways involved in homogentisic acid synthesis are also responsible for tyrosine formation, any alterations in these pathways can also result in the alteration in tyrosine synthesis and the synthesis of other aromatic amino acids.

Synthesis of Phytylpyrophosphate

Tocopherols are a member of the class of compounds referred to as the isoprenoids. Other isoprenoids include carotenoids, gibberellins, terpenes, chlorophyll and abscisic acid. A central intermediate in the production of isoprenoids is isopentenyl diphosphate (IPP). Cytoplasmic and plastid-based pathways to generate IPP have been reported. The cytoplasmic based pathway involves the enzymes acetoacetyl CoA thiolase, HMGCoA synthase, HMGCoA reductase, mevalonate kinase, phosphomevalonate kinase, and mevalonate pyrophosphate decarboxylase.

Recently, evidence for the existence of an alternative, plastid based, isoprenoid biosynthetic pathway emerged from studies in the research groups of Rohmer and Arigoni (Eisenreich *et al.*, *Chem. Bio.*, 5:R221-R233, 1998); Rohmer, *Prog. Drug. Res.*, 50:135-154, 1998); Rohmer, *Comprehensive Natural Products Chemistry*, Vol. 2, pp. 45-68, Barton and Nakanishi (eds.), Pergamon Press, Oxford, England (1999), who found that the isotope labeling patterns observed in studies on certain eubacterial and plant terpenoids could not be explained in terms of the mevalonate pathway. Arigoni and coworkers subsequently showed that 1-deoxyxylulose, or a derivative thereof, serves as an intermediate of the novel pathway, now referred to as the MEP pathway (Rohmer *et al.*, *Biochem. J.*, 295:517-524, 1993); Schwarz, Ph.D. thesis, Eidgenössiche Technische Hochschule, Zurich, Switzerland, 1994). Recent studies showed the formation of 1-deoxyxylulose 5-phosphate (Broers, Ph.D. thesis (Eidgenössiche Technische Hochschule, Zurich, Switzerland) (1994) from one molecule each of glyceraldehyde 3-phosphate (Rohmer, *Comprehensive Natural Products Chemistry*, Vol. 2, pp. 45-68, Barton and Nakanishi, eds., Pergamon Press, Oxford, England (1999) and pyruvate (Eisenreich *et al.*, *Chem. Biol.*, 5:R223-R233, 1998); Schwarz *supra*; Rohmer *et al.*, *J. Am. Chem. Soc.*, 118:2564-2566 (1996); and Sprenger *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 94:12857-12862, 1997) by an enzyme encoded by the *dxs* gene (Lois *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 95:2105-2110, 1997; U.S. Patent Publication 2003/0125573); and

Lange *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 95:2100-2104, 1998). 1-Deoxyxylulose 5-phosphate can be further converted into 2-*C*-methylerythritol 4-phosphate (Arigoni *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 94:10600-10605, 1997) by a reductoisomerase encoded by the *dxr* gene (Bouvier *et al.*, *Plant Physiol.*, 117:1421-1431, 1998); and Rohdich *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 96:11758-11763, 1999).

Genes reported to be in the MEP pathway also include *ygbP,* which catalyzes the conversion of 2-C-methyl-D-erythritol 4-phosphate into its respective cytidyl pyrophosphate derivative. The translation product of *chB,* in turn catalyzes the conversion of 4-phosphocytidyl-2C-methyl-D-erythritol into 4-diphosphocytidyl-2C-methyl-D-erythritol-2 phosphate. The latter compound is converted by the action of the translation product of ygbB into 2-C-methyl-D-erythritol, 2, 4-cyclophosphate. Subsequently, 2C-methyl-D-erythritol, 2, 4-cyclophosphate is converted by the translation product of gcpE to (E)-1-(4-hydroxy-3-methylbut-2-enyl) diphosphate. The latter compound is converted by the action of LytB to IPP and DMAPP (Herz *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 97(6):2485-2490, 2000).

Once IPP is formed by the MEP pathway, it is converted to GGDP by GGDP synthase, and then to phytylpyrophosphate, which is the central constituent of the tocopherol side chain.

Combination and Cyclization

Homogentisic acid is combined with either phytylpyrophosphate or solanyl-pyrophosphate by phytyl/prenyl transferase forming 2-methylphytyl plastoquinol or 2-methylsolanyl plastoquinol, respectively. 2-Methylsolanyl plastoquinol is a precursor to the biosynthesis of plastoquinones, while 2-methylphytyl plastoquinol is ultimately converted to tocopherol. It has been suggested that homogentisic acid, when combined with geranylgeranylpyrophosphate, will lead to the formation of tocotrienols.

Methylation of the Aromatic Ring

The major structural difference between each of the tocopherol subtypes is the position of the methyl groups around the phenyl ring. Both 2-methylphytyl plastoquinol and 2-methylsolanyl plastoquinol serve as substrates for the plant enzyme 2-methylphytylplastoquinol/2-methylsolanylplastoquinol methyltransferase (Tocopherol Methyl Transferase 2; Methyl Transferase 2; MT2; tMT2), which is capable of methylating a tocopherol precursor. Subsequent methylation of γ-tocopherol by γ-tocopherol methyl-transferase (GMT) generates the biologically active α-tocopherol.

A possible alternate pathway for the generation of α-tocopherol involves the generation of δ-tocopherol via the cyclization of 2-methylphytylplastoquinol by tocopherol cyclase. δ-tocopherol is then converted to β-tocopherol via the methylation of the 5 position by GMT. δ-tocopherol can be converted to α-tocopherol via methylation of the 3 position by tMT2, followed by methylation of the 5 position by GMT. In a possible alternative pathway, β-tocopherol is directly converted to α-tocopherol by tMT2 via the methylation of the 3 position (see, for example, *Biochemical Society Transactions*, 11:504-510 (1983); *Introduction to Plant Biochemistry*, 2nd edition, chapter 11 (1983); *Vitamin Hormone*, 29:153-200, 1971); *Biochemical Journal*, 109:577 (1968); and, *Biochemical and Biophysical Research Communication*, 28(3):295 (1967). Since all potential mechanisms for the generation of α-tocopherol involve catalysis by tMT2, plants that are deficient in this activity accumulate δ-tocopherol and β-tocopherol. Plants that have increased tMT2 activity tend to accumulate γ-tocopherol and α-tocopherol. Since there is a low level of GMT activity in the seeds of many plants, these plants tend to accumulate γ-tocopherol.

The agents of the invention will preferably be "biologically active" with respect to either a structural attribute, such as the capacity of a nucleic acid to hybridize to another nucleic acid molecule, or the ability of a protein to be bound by an antibody (or to compete with another molecule for such binding). Alternatively, such an attribute may be catalytic and thus involve the capacity of the agent to mediate a chemical reaction or response. The agents will preferably be "substantially purified." The term "substantially purified," as used herein, refers to a molecule separated from substantially all other molecules normally associated with it in its native environmental conditions. More preferably a substantially purified molecule is the predominant species present in a preparation. A substantially purified molecule may be greater than 60% free, preferably 75% free, more preferably 90% free, and most preferably 95% free from the other molecules (exclusive of solvent) present in the natural mixture. The term "substantially purified" is not intended to encompass molecules present in their native environmental conditions.

The agents of the invention may also be recombinant. As used herein, the term recombinant means any agent (*e.g.*, DNA, peptide, *etc.*), that is, or results, however indirectly, from human manipulation of a nucleic acid molecule.

It is understood that the agents of the invention may be labeled with reagents that facilitate detection of the agent (*e.g.*, fluorescent labels, Prober *et al.*, *Science*, 238:336-340

(1987); Albarella *et al.*, EP 144914; chemical labels, Sheldon *et al.*, U.S. Patent 4,582,789; Albarella *et al.*, U.S. Patent 4,563,417; modified bases, Miyoshi *et al.*, EP 119448).

Tocopherols are plant chloroplast lipophilic molecules involved in the response of plants to oxidative stresses (Porfirova *et al*, *PNAS*, 99(19):12495-12500, 2002). Therefore, in another preferred embodiment, expression or over-expression of a phytol kinase or polypeptide having phytol kinase activity (SEQ ID NOs: 2, 6, and 20-28) (Figure 1) of the present invention in a transformed plant may provide tolerance to a variety of stresses, *e.g.*, oxidative stress tolerance such as to drought, oxygen or ozone, UV tolerance, cold tolerance, or fungal/microbial pathogen tolerance. Environmental stresses, such as drought, increased salinity of soil, and extreme temperature, are major factors in limiting plant growth and productivity. The worldwide loss in yield of three major cereal crops, rice, maize (corn), and wheat due to water stress (drought) has been estimated to be over ten billion dollars annually. However, conventional breeding is a slow process for generating crop varieties with improved tolerance to stress conditions. Limited germplasm resources for stress tolerance and incompatibility in crosses between distantly related plant species are additional problems encountered in conventional breeding. Recent progress in plant genetic transformation and availability of potentially useful genes characterized from different sources make it possible to generate stress-tolerant crops using transgenic approaches (U.S. Patent 5,981,842).

As used herein in a preferred aspect, a tolerance or resistance to stress is determined by the ability of a plant, when challenged by a stress such as drought to produce a plant having a higher yield or to a plant being less susceptible to an environmentally induced phenotype such as wilting, than one without such tolerance or resistance to stress. In a particularly preferred aspect of the present invention, the tolerance or resistance to stress is measured relative to a plant with a similar genetic background to the tolerant or resistance plant except that the plant expresses or over expresses a protein or fragment thereof of the present invention.

Nucleic Acid Molecules

The present invention includes and provides nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 30-41 and 53-68.

The present invention includes and provides nucleic acid molecules encoding a phytol kinase polypeptide, or a polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 6, 20-68, and 79 or

comprising an amino acid sequence having at least about 70, 80, 90, 95, or 99% identity to such amino acid sequences.

The present invention includes and provides nucleic acid molecules comprising a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1, 5, and 17 and sequences having at least about 70, 80, 90, 95, or 99% identity to such sequences.

The present invention includes and provides nucleic acid molecules encoding a phytol kinase polypeptide, or a polypeptide having phytol kinase activity.

The present invention includes and provides nucleic acid molecules encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity.

The present invention includes and provides nucleic acid molecules encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity.

The present invention includes and provides nucleic acid molecules encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 6, and 37-68.

The present invention includes and provides nucleic acid molecules encoding cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-27, 29-34, and 79.

The present invention includes and provides nucleic acid molecules encoding a yeast phytol kinase polypeptide, or a yeast polypeptide having phytol kinase activity.

The present invention includes and provides nucleic acid molecules encoding a yeast phytol kinase polypeptide, or a yeast polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 35 and 36.

The present invention includes and provides nucleic acid molecules encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid selected from the group consisting of SEQ ID NOs: 74, 77, and 78.

The present invention includes and provides nucleic acid molecules encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid selected from the group consisting of SEQ ID NOs: 74, 77, and 78, wherein said polypeptide is not derived from *Allium porrum, Brassica napus, Gossypium, Glycine max, Oryza sativa, Sorghum bicolor, Triticum aestivum,* and *Zea mays*

The present invention includes and provides nucleic acid molecules encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 74, 77, and 78 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 75 and 76.

The present invention includes and provides nucleic acid molecules encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 74, 77, and 78 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 75 and 76, wherein said polypeptide is not derived from *Allium porrum, Brassica napus, Gossypium, Glycine max, Oryza sativa, Sorghum bicolor, Triticum aestivum,* and *Zea mays.*

The present invention includes and provides nucleic acid molecules encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 71, 72, and 73.

The present invention includes and provides nucleic acid molecules encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 71, 72, and 73, wherein said polypeptide is not derived from *Synechocystis, Aquifex aeolicus, Chlorobium tepidum, Chloroflexus aurantiacus, Nostoc punctiforme, Prochlorococcus marinus, Rickettsia conorii, Rickettsia prowazekii, Rickettsia sibirica, Synechoccus, Thermosynechoccus elongatus, Trichodesmium erythraeum* and *Saccharomyces cerevisiae.*

The present invention includes and provides nucleic acid molecules encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising one or more amino acid sequences selected from the group consisting of SEQ ID NOs: 71, 72, and 73 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 69 and 70.

The present invention includes and provides nucleic acid molecules encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising one or more amino acid sequences selected from the group consisting of SEQ ID NOs: 71, 72, and 73 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 69 and 70, wherein said polypeptide is not derived

from *Synechocystis, Aquifex aeolicus, Chlorobium tepidum, Chloroflexus aurantiacus, Nostoc punctiforme, Prochlorococcus marinus, Rickettsia conorii, Rickettsia prowazekii, Rickettsia sibirica, Synechoccus, Thermosynechoccus elongatus, Trichodesmium erythraeum,* and *Saccharomyces cerevisiae.*

In another preferred aspect of the present invention a nucleic acid molecule comprises nucleotide sequences encoding a plastid transit peptide operably fused to a nucleic acid molecule that encodes a protein or fragment of the present invention.

It is understood that in a further aspect of nucleic acid sequences of the present invention, the nucleic acids can encode a protein that differs from any of the proteins in that one or more amino acids have been deleted, substituted or added without altering the function. For example, it is understood that codons capable of coding for such conservative amino acid substitutions are known in the art.

In one aspect of the present invention the nucleic acids of the present invention are said to be introduced nucleic acid molecules. A nucleic acid molecule is said to be "introduced" if it is inserted into a cell or organism as a result of human manipulation, no matter how indirect. Examples of introduced nucleic acid molecules include, without limitation, nucleic acids that have been introduced into cells via transformation, transfection, injection, and projection, and those that have been introduced into an organism via conjugation, endocytosis, phagocytosis, etc.

One subset of the nucleic acid molecules of the invention is fragment nucleic acids molecules. Fragment nucleic acid molecules may consist of significant portion(s) of, or indeed most of, the nucleic acid molecules of the invention, such as those specifically disclosed. Alternatively, the fragments may comprise smaller oligonucleotides (having from about 15 to about 400 nucleotide residues and more preferably, about 15 to about 30 nucleotide residues, or about 50 to about 100 nucleotide residues, or about 100 to about 200 nucleotide residues, or about 200 to about 400 nucleotide residues, or about 275 to about 350 nucleotide residues).

A fragment of one or more of the nucleic acid molecules of the invention may be a probe and specifically a PCR probe. A PCR probe is a nucleic acid molecule capable of initiating a polymerase activity while in a double-stranded structure with another nucleic acid. Various methods for determining the structure of PCR probes and PCR techniques exist in the art.

Nucleic acid molecules or fragments thereof of the present invention are capable of specifically hybridizing to other nucleic acid molecules under certain circumstances. Nucleic acid molecules of the present invention include those that specifically hybridize to nucleic acid molecules having a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 17, and complements thereof. Nucleic acid molecules of the present invention also include those that specifically hybridize to nucleic acid molecules encoding an amino acid sequence selected from SEQ ID NOs: 2, 6, 20-68, and 79, and fragments thereof.

As used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure.

A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are described by Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press*, Cold Spring Harbor, New York (2001), and by Haymes *et al., Nucleic Acid Hybridization, A Practical Approach, IRL Press*, Washington, DC (1985). Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. Thus, in order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

Appropriate stringency conditions which promote DNA hybridization are, for example, 6.0 X sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 X SSC at 20-25°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 X SSC at 50°C to a high stringency of about 0.2 X SSC at 65°C. In addition, the temperature in

the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C. Both temperature and salt may be varied, or either the temperature or the salt concentration may be held constant while the other variable is changed.

In a preferred embodiment, a nucleic acid of the present invention will specifically hybridize to one or more of the nucleic acid molecules set forth in SEQ ID NOs: 1, 3, 5, and 17, and complements thereof under moderately stringent conditions, for example at about 2.0 X SSC and about 65°C.

In a particularly preferred embodiment, a nucleic acid of the present invention will include those nucleic acid molecules that specifically hybridize to one or more of the nucleic acid molecules set forth in SEQ ID NOs: 1, 3, 5, and 17, and complements thereof under high stringency conditions such as 0.2 X SSC and about 65°C.

In one embodiment of a method of the present invention, any of the nucleic acid sequences or polypeptide sequences, or fragments of either, of the present invention can be used to search for related sequences. In a preferred embodiment, a member selected from the group consisting of SEQ ID NOs: 69-78 is used to search for related sequences. In another embodiment, any of the motifs or regions of conserved sequence shown in Figures 11-20 are used to search for related amino acid sequences. In one embodiment, one or more of SEQ ID NOs: 74, 77, and 78, and one or more of SEQ ID NOs: 75 and 76 are used to search for related sequences. In one embodiment, one or more of SEQ ID NOs: 71, 72 and 73 are used to search for related sequences. As used herein, "search for related sequences" means any method of determining relatedness between two sequences, including, but not limited to, searches that compare sequence homology: for example, a PBLAST search of a database for relatedness to a single amino acid sequence. Other searches may be conducted using profile based methods, such as the HMM (Hidden Markov model) META-MEME (http://metameme.sdsc.edu/mhmm-links.html), PSI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The present invention includes and provides for phytol kinases discovered using one or more of the alignments of Figures 11-20.

A polypeptide or polynucleotide molecule can be substantially identical or substantially homologous to related molecules. These homologues with substantial identity to a related molecule generally comprise at least one polypeptide sequence or one polynucleotide sequence that has at least seventy percent sequence identity compared to other polypeptide sequences or polynucleotide sequences. The Gap program in the WISCONSIN

PACKAGE version 10.0-UNIX from Genetics Computer Group, Inc. based on the method of Needleman and Wunsch (J. Mol. Biol. 48:443-453, 1970) using the set of default parameters for pairwise comparison (for amino acid sequence comparison: Gap Creation Penalty = 8, Gap Extension Penalty = 2; for nucleotide sequence comparison: Gap Creation Penalty = 50; Gap Extension Penalty = 3) or using the TBLASTN program in the BLAST 2.2.1 software suite (Altschul *et al.*, Nucleic Acids Res. 25:3389-3402), using BLOSUM62 matrix (Henikoff and Henikoff, Proc. Natl. Acad. Sci. U.S.A. 89:10915-10919, 1992) and the set of default parameters for pair-wise comparison (gap creation cost = 11, gap extension cost = 1.). In BLAST, the E-value, or expectation value, represents the number of different alignments with scores equivalent to or better than the raw alignment score, S, that are expected to occur in a database search by chance. The lower the E value, the more significant the match. Because database size is an element in E-value calculations, E-values obtained by "BLASTing" against public databases, such as GenBank, have generally increased over time for any given query/entry match. Percent identity refers to the percentage of identically matched amino acid residues that exist along the length of that portion of the sequences which is aligned by the BLAST algorithm. In a preferred embodiment the percent identity calculations are performed using BLASTN or BLASTP (default, parameters, version 2.0.8, Altschul *et al., Nucleic Acids Res.*, 25:3389-3402 (1997).

A nucleic acid molecule of the invention can also encode a homolog polypeptide. As used herein, a homolog polypeptide molecule or fragment thereof is a counterpart protein molecule or fragment thereof in a second species (*e.g.*, corn rubisco small subunit is a homolog of *Arabidopsis* rubisco small subunit). A homolog can also be generated by molecular evolution or DNA shuffling techniques, so that the molecule retains at least one functional or structure characteristic of the original polypeptide (*see*, for example, U.S. Patent 5,811,238).

Agents of the invention include nucleic acid molecules that encode having at least about a contiguous 10 amino acid region of a polypeptide of the present invention, more preferably having at least about a contiguous 25, 40, 50, 100, or 125 amino acid region of a polypeptide of the present invention, preferably a polypeptide comprising SEQ ID NO: 2, 6, or 20-68.

In a preferred embodiment, any of the nucleic acid molecules of the present invention can be operably linked to a promoter region that functions in a plant cell to cause the production of an mRNA molecule, where the nucleic acid molecule that is linked to the

promoter is heterologous with respect to that promoter. As used herein, "heterologous" means not naturally occurring together.

Protein and Peptide Molecules

A class of agents includes one or more of the polypeptide molecules encoded by a nucleic acid agent of the invention. A particular preferred class of proteins is that having an amino acid sequence of SEQ ID NOs 2, 6, or 20-68, or a sequence having at least about 70, 80, 90, 95 or 99% identity to such sequences, or fragments thereof.

In another aspect of the present invention, the polypeptide is a phytol kinase or a polypeptide having phytol kinase activity. In another aspect of the present invention, the polypeptide is a plant, cyanobacterial, or yeast polypeptide. In still another aspect of the present invention, the phytol kinase polypeptide, or a polypeptide having phytol kinase activity, comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 6, 20-68, and 79. In still another aspect of the present invention, the polypeptide is a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 6, and 37-68.

In still another aspect of the present invention, the polypeptide is a yeast phytol kinase polypeptide, or a yeast polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SDQ ID NOs: 35 and 36.

In one embodiment of the present invention, the polypeptide is a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-27, 29-34, and 79. The present invention includes and provides plant phytol kinase polypeptides, or plant polypeptides having phytol kinase activity, comprising an amino acid selected from the group consisting of SEQ ID NOs: 74, 77, and 78. In another aspect of the present invention, the plant phytol kinase polypeptide, or plant polypeptide having phytol kinase activity, comprises an amino acid selected from the group consisting of SEQ ID NOs: 74, 77, and 78, wherein said polypeptide is not derived from *Allium porrum, Brassica napus, Gossypium, Glycine max, Oryza sativa, Sorghum bicolor, Triticum aestivum,* and *Zea mays.*

In yet another aspect of the present invention, the plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 74, 77, and 78 and further comprises an amino

acid sequence comprising one or more of SEQ ID NOs: 75 and 76. The present invention includes and provides plant phytol kinase polypeptides, or plant polypeptides having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 74, 77, and 78 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 75 and 76, wherein said polypeptide is not derived from *Allium porrum, Brassica napus, Gossypium, Glycine max, Oryza sativa, Sorghum bicolor, Triticum aestivum,* and *Zea mays.*

The present invention includes and provides cyanobacterial phytol kinase polypeptides, or cyanobacterial polypeptides having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 71, 72, and 73. The present invention includes and provides cyanobacterial phytol kinase polypeptides, or cyanobacterial polypeptides having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 71, 72, and 73, wherein said polypeptide is not derived from *Synechocystis, Aquifex aeolicus, Chlorobium tepidum, Chloroflexus aurantiacus, Nostoc punctiforme, Prochlorococcus marinus, Rickettsia conorii, Rickettsia prowazekii, Rickettsia sibirica, Synechoccus, Thermosynechoccus elongatus, Trichodesmium erythraeum* and *Saccharomyces cerevisiae.* In another aspect of the present invention, a class of proteins includes cyanobacterial phytol kinase polypeptides, or cyanobacterial polypeptides having phytol kinase activity, comprising one or more amino acid sequences selected from the group consisting of SEQ ID NOs: 71, 72, and 73 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 69 and 70.

The present invention includes and provides cyanobacterial phytol kinase polypeptides, or cyanobacterial polypeptides having phytol kinase activity, comprising one or more amino acid sequences selected from the group consisting of SEQ ID NOs: 71, 72, and 73 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 69 and 70, wherein said polypeptide is not derived from *Synechocystis, Aquifex aeolicus, Chlorobium tepidum, Chloroflexus aurantiacus, Nostoc punctiforme, Prochlorococcus marinus, Rickettsia conorii, Rickettsia prowazekii, Rickettsia sibirica, Synechoccus, Thermosynechoccus elongatus, Trichodesmium erythraeum,* and *Saccharomyces cerevisiae.*

Polypeptide agents may have C-terminal or N-terminal amino acid sequence extensions. One class of N-terminal extensions employed in a preferred embodiment are plastid transit peptides. When employed, plastid transit peptides can be operatively linked to the N-terminal sequence, thereby permitting the localization of the agent polypeptides to

plastids. In an embodiment of the present invention, any suitable plastid targeting sequence can be used (see, *e.g.,* U.S. Patents 5,776,760; 6,489,542; and 5,717,084). Where suitable, a plastid targeting sequence can be substituted for a native plastid targeting sequence. In a further embodiment, any suitable, modified plastid targeting sequence can be used. In another embodiment, *e.g.,* the plastid targeting sequence is a CTP1 sequence (U.S. Patent 5,776,760).

As used herein, the term "protein," "peptide molecule," or "polypeptide" includes any molecule that comprises five or more amino acids. It is well known in the art that protein, peptide or polypeptide molecules may undergo modification, including post-translational modifications, such as, but not limited to, disulfide bond formation, glycosylation, phosphorylation, or oligomerization. Thus, as used herein, the term "protein," "peptide molecule," or "polypeptide" includes any protein that is modified by any biological or non-biological process. The terms "amino acid" and "amino acids" refer to all naturally occurring L-amino acids. This definition is meant to include norleucine, norvaline, ornithine, homocysteine, and homoserine.

A "protein fragment" is a peptide or polypeptide molecule whose amino acid sequence comprises a subset of the amino acid sequence of that protein. A protein or fragment thereof that comprises one or more additional peptide regions not derived from that protein is a "fusion" protein. Such molecules may be derivatized to contain carbohydrate or other moieties (such as keyhole limpet hemocyanin). Fusion protein or peptide molecules of the invention are preferably produced via recombinant means.

Plant Constructs and Plant Transformants

One or more of the nucleic acid molecules of the invention may be used in plant transformation or transfection. Exogenous genetic material may be transferred into a plant cell and the plant cell regenerated into a whole, fertile or sterile plant. Exogenous genetic material is any genetic material, whether naturally occurring or otherwise, from any source that is capable of being inserted into any organism.

In a preferred aspect of the present invention the exogenous genetic material comprises a nucleic acid sequence of SEQ ID NOs: 1, 5, 17, or nucleic acid sequences having at least about 70, 80, 90, 95, or 99% identity to such sequences or complements thereof and fragments of either. In a further aspect of the present invention the exogenous genetic material comprises a nucleic acid sequence encoding an amino acid sequence selected from

the group consisting of SEQ ID NOs: 2, 6, 20-68, and 79, sequences having at least about 70, 80, 90, 95 or 99% identity to such sequences, or fragments thereof.

In another aspect of the present invention, the exogenous genetic material comprises nucleic acid molecules encoding a phytol kinase polypeptide or polypeptide having phytol kinase activity.

In another aspect of the present invention, the exogenous genetic material comprises nucleic acid molecules encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity.

In another aspect of the present invention, the exogenous genetic material comprises nucleic acid molecules encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity.

In another aspect of the present invention, the exogenous genetic material comprises nucleic acid molecules encoding a phytol kinase polypeptide, or a polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 6, 20-68, and 79.

In another aspect of the present invention, the exogeneous genetic material comprises nucleic acid molecules encoding a yeast phytol kinase polypeptide, or a yeast polypeptide having phytol kinase activity.

In another aspect of the present invention, the exogeneous genetic material comprises nucleic acid molecules encoding a yeast phytol kinase polypeptide, or a yeast polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 35 and 36.

In another aspect of the present invention, the exogenous genetic material comprises nucleic acid molecules encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 6, and 37-68.

In another aspect of the present invention, the exogenous genetic material comprises nucleic acid molecules encoding cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-27, 29-34, and 79.

In another aspect of the present invention, the exogenous genetic material comprises nucleic acid molecules encoding a plant phytol kinase polypeptide, or a plant polypeptide

having phytol kinase activity, comprising an amino acid selected from the group consisting of SEQ ID NOs: 74, 77, and 78.

In another aspect of the present invention, the exogenous genetic material comprises nucleic acid molecules encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid selected from the group consisting of SEQ ID NOs: 74, 77, and 78, wherein said polypeptide is not derived from *Allium porrum, Brassica napus, Gossypium, Glycine max, Oryza sativa, Sorghum bicolor, Triticum aestivum,* and *Zea mays.*

In another aspect of the present invention, the exogenous genetic material comprises nucleic acid molecules encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 74, 77, and 78 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 75 and 76.

In another aspect of the present invention, the exogenous genetic material comprises nucleic acid molecules encoding a plant phytol kinase polypeptide, or a plant polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 74, 77, and 78 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 75 and 76, wherein said polypeptide is not derived from *Allium porrum, Brassica napus, Gossypium, Glycine max, Oryza sativa, Sorghum bicolor, Triticum aestivum,* and *Zea mays.*

In another aspect of the present invention, the exogenous genetic material comprises nucleic acid molecules encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 71, 72, and 73.

In another aspect of the present invention, the exogenous genetic material comprises nucleic acid molecules encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 71, 72, and 73, wherein said polypeptide is not derived from *Synechocystis, Aquifex aeolicus, Chlorobium tepidum, Chloroflexus aurantiacus, Nostoc punctiforme, Prochlorococcus marinus, Rickettsia conorii, Rickettsia prowazekii, Rickettsia sibirica, Synechoccus, Thermosynechoccus elongatus, Trichodesmium erythraeum* and *Saccharomyces cerevisiae.*

In another aspect of the present invention, the exogenous genetic material comprises a nucleic acid molecule encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising one or more amino acid sequences selected from the group consisting of SEQ ID NOs: 71, 72, and 73 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 69 and 70.

In another aspect of the present invention, the exogenous genetic material comprises a nucleic acid molecule encoding a cyanobacterial phytol kinase polypeptide, or a cyanobacterial polypeptide having phytol kinase activity, comprising one or more amino acid sequences selected from the group consisting of SEQ ID NOs: 71, 72, and 73 and further comprising an amino acid sequence comprising one or more of SEQ ID NOs: 69 and 70, wherein said polypeptide is not derived from *Synechocystis, Aquifex aeolicus, Chlorobium tepidum, Chloroflexus aurantiacus, Nostoc punctiforme, Prochlorococcus marinus, Rickettsia conorii, Rickettsia prowazekii, Rickettsia sibirica, Synechoccus, Thermosynechoccus elongatus, Trichodesmium erythraeum,* and *Saccharomyces cerevisiae.*

In a further aspect of the present invention, the nucleic acid sequences of the invention also encode peptides involved in intracellular localization, export, or post-translational modification.

In an embodiment of the present invention, exogenous genetic material encoding an LTT1 or fragment thereof is introduced into a plant with one or more additional genes. In one embodiment, preferred combinations of genes include a nucleic acid molecule of the present invention and one or more of the following genes: tyrA (*e.g.,* WO 02/089561 and Xia *et al., J. Gen. Microbiol.,* 138:1309-1316, 1992), tocopherol cyclase (*e.g.,* WO 01/79472), prephenate dehydrogenase, dxs (*e.g.,* Lois *et al., Proc. Natl. Acad. Sci. (U.S.A.),* 95(5):2105-2110, 1998), dxr (*e.g.,* U.S. Publication 2002/0108814A and Takahashi *et al., Proc. Natl. Acad. Sci. (U.S.A.),* 95 (17), 9879-9884, 1998), GGPPS (*e.g.,* Bartley and Scolnik, *Plant Physiol.,* 104:1469-1470, 1994), HPPD (*e.g.,* Norris *et al., Plant Physiol.,* 117:1317-1323, 1998; U.S. Patent 6,087,563), GMT (*e.g.,* U.S. Patent Appn. 10/219,810, filed August 16, 2002; WO 03/016482), HPT (U.S. Patent 6,541,259) (tMT2 (*e.g.,* U.S. Patent Application 10/279,029, filed October 24, 2002; WO 03/034812), AANT1 (*e.g.,* WO 02/090506), IDI (E.C.:5.3.3.2; Blanc *et al.,* In: *Plant Gene Register,* PRG96-036; and Sato *et al.,* DNA Res., 4:215-230, 1997), GGH (Graβes *et al., Planta.,* 213-620, 2001), or a plant ortholog and an antisense construct for homogentisic acid dioxygenase (Kridl *et al., Seed Sci. Res.,* 1:209-219, 1991); Keegstra, *Cell,* 56(2):247-53, 1989); Nawrath, *et al., Proc. Natl.*

Acad. Sci. (U.S.A.), 91:12760-12764, 1994); Cyanobase, www.kazusa.or.jp/cyanobase; Smith *et al., Plant J.*, 11:83-92, 1997); WO 00/32757; ExPASy Molecular Biology Server, http://us.expasy.org/enzyme; MT1 (*e.g.*, WO 00/10380); gcpE (*e.g.* WO 02/12478); Saint Guily *et al., Plant Physiol.*, 100(2):1069-1071, 1992); Sato *et al., J. DNA Res.*, 7(1):31-63, 2000). In such combinations, in some crop plants, *e.g.*, canola, a preferred promoter is a napin promoter and a preferred plastid targeting sequence is a CTP1 sequence. It is preferred that gene products are targeted to the plastid. Alternatively, one or more of the gene products can be localized in the cytoplasm. In a preferred aspect, the gene products of *tyrA* and HPPD are targeted to the plastids. In a second preferred embodiment, TyrA and HPPD are targeted to the cytoplasm. Such genes can be introduced, for example, on a single construct, introduced on different constructs but the same transformation event, or introduced into separate plants followed by one or more crosses to generate the desired combination of genes. In such combinations, a preferred promoter is a napin, 7S alpha promoter, the 7S alpha' promoter, the Arcelin 5 promoter, the USP88 promoter and a preferred plastid targeting sequence is a CTP1 sequence. It is preferred that gene products are targeted to the plastid.

In a preferred combination, a nucleic acid molecule of the present invention and a nucleic acid molecule encoding any of the following enzymes: *tyrA,* HPT *slr1736, tocopherol cyclase, chlorophyllase, dxs, dxr, GGPPS, HPPD, tMT2, AANT1, slr1737, IDI, GGH* or a plant ortholog and an antisense construct for homogentisic acid dioxygenase are introduced into a plant.

Such genetic material may be transferred into either monocotyledons or dicotyledons including, but not limited to canola, corn, soybean, *Arabidopsis, Phaseolus*, peanut, alfalfa, wheat, rice, oat, sorghum, rapeseed, rye, tritordeum, millet, fescue, perennial ryegrass, sugarcane, cranberry, papaya, banana, safflower, oil palms, flax, muskmelon, apple, cucumber, dendrobium, gladiolus, chrysanthemum, liliacea, cotton, eucalyptus, sunflower, *Brassica campestris,* oilseed rape, turfgrass, sugarbeet, coffee and dioscorea (Christou, In: *Particle Bombardment for Genetic Engineering of Plants,* Biotechnology Intelligence Unit. Academic Press, San Diego, California (1996), with canola, corn, *Brassica campestris,* oilseed rape, rapeseed, soybean, crambe, mustard, castor bean, peanut, sesame, cottonseed, linseed, safflower, oil palm, flax, and sunflower preferred, and canola, rapeseed, corn, *Brassica campestris,* oilseed rape, soybean, sunflower, safflower, oil palms, and peanut preferred. In a preferred embodiment, the homolog is selected from the group consisting of maize, soybean, canola, cottonseed, sesame, flax, peanut, sunflower, safflower, and oil palm.

In a more preferred embodiment, the genetic material is transferred into canola. In another more preferred embodiment, the genetic material is transferred into oilseed rape. In another particularly preferred embodiment, the genetic material is transferred into soybean.

Transfer of a nucleic acid molecule that encodes a protein can result in expression or overexpression of that polypeptide in a transformed cell or transgenic plant. One or more of the proteins or fragments thereof encoded by nucleic acid molecules of the invention may be overexpressed in a transformed cell or transformed plant. Such expression or overexpression may be the result of transient or stable transfer of the exogenous genetic material.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, 99% identity to such sequences provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, 99% identity to such sequences provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of α-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, 99% identity to such sequences provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of γ-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, 99% identity to such sequences provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of δ-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, 99% identity to such sequences provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of β-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, 99% identity to such sequences provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, 99% identity to such sequences provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of α-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, 99% identity to such sequences provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of γ-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, 99% identity to such sequences provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of δ-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, 99% identity to such sequences provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of β-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, 99% identity to such sequences provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of plastoquinols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or polypeptide having phytol kinase activity provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or a polypeptide having phytol kinase activity, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of α-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or a polypeptide having phytol kinase activity, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of γ-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or a polypeptide having phytol kinase activity, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of β-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or a polypeptide having phytol kinase activity, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of δ-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide, or a polypeptide having phytol kinase activity, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide, or a polypeptide having phytol kinase activity, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of α-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide, or a polypeptide having phytol kinase activity, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of γ-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or a polypeptide having phytol kinase activity, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of β-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or a polypeptide having phytol kinase activity, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of δ-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed plant,

relative to an untransformed plant with a similar genetic background, an increased level of tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of α-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of γ-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of β-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of δ-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of α-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of γ-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of β-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of δ-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding polypeptides of the present invention provide in a transformed plant, relative to an untransformed plant with a similar genetic background, an increased level of plastoquinols.

In one embodiment, DNA constructs of the present invention comprising SEQ ID NO: 3 provide in a transformed plant, relative to an untransformed plant with a similar genetic background, a decreased level of tocopherols, α-tocopherols, γ-tocopherols, δ-tocopherols, β-tocopherols, tocotrienols, α-tocotrienols, γ-tocotrienols, δ-tocotrienols, β-tocotrienols, and/or plastoquinols.

In any of the embodiments described herein, an increase in γ-tocopherol, α-tocopherol, or both can lead to a decrease in the relative proportion of β-tocopherol, δ-tocopherol, or both. Similarly, an increase in γ-tocotrienol, α-tocotrienol, or both can lead to a decrease in the relative proportion of β-tocotrienol, δ-tocotrienol, or both.

In some embodiments, the levels of one or more products of the tocopherol biosynthesis pathway, including any one or more of tocopherols, α -tocopherols, γ-tocopherols, δ-tocopherols, β-tocopherols, tocotrienols, α-tocotrienols, γ-tocotrienols, δ-tocotrienols, β-tocotrienols are measurably increased. The levels of products may be

increased throughout an organism such as a plant or localized in one or more specific organs or tissues of the organism. For example the levels of products may be increased in one or more of the tissues and organs of a plant including without limitation: roots, tubers, stems, leaves, stalks, fruit, berries, nuts, bark, pods, seeds and flowers. A preferred organ is a seed.

In a preferred embodiment, expression of enzymes involved in tocopherol, tocotrienol or plastoquinol synthesis in the seed will result in an increase in γ-tocopherol levels due to the absence of significant levels of GMT activity in those tissues. In another preferred embodiment, expression of enzymes involved in tocopherol, tocotrienol or plastoquinol synthesis in photosynthetic tissues will result in an increase in α-tocopherol due to the higher levels of GMT activity in those tissues relative to the same activity in seed tissue.

In another preferred embodiment, the expression of enzymes involved in tocopherol, tocotrienol or plastoquinol synthesis in the seed will result in an increase in the total tocopherol, tocotrienol or plastoquinol level in the plant.

In some embodiments, the levels of tocopherols or a species such as α-tocopherol may be altered. In some embodiments, the levels of tocotrienols may be altered. Such alteration can be compared to a plant with a similar genetic background but lacking the introduction of a nucleic acid sequence of the present invention.

In another embodiment, either the α-tocopherol level, α-tocotrienol level, or both of plants that natively produce high levels of either α-tocopherol, α-tocotrienol or both (*e.g.*, sunflowers), can be increased by the introduction of a nucleic acid of the present invention.

As tocotrienols have their own health benefits, the nucleotide sequence of LTT1 and nucleotide sequences encoding phytol kinase polypeptides and polypeptides having phytol kinase activity can also be used to obtain transgenic seed that predominantly accumulate tocotrienols. Tocotrienols can be obtained in dicotyledone seed that carry seed-specific expression constructs for the prephenate dehydrogenase (tyrA) and the p-hydroxyphenylpyruvate dioxygenase (HPPD) (WO 02/089561). A higher purity of tocotrienols may be obtained in such seed by reducing the production of tocopherols while increasing the production of tocotrienols. Tocopherol biosynthesis can be reduced by a mutation in LTT1. Alternatively tocopherol biosynthesis may be reduced by downregulating LTT1 and other nucleotide sequences encoding phytol kinase polypeptides and polypeptides having phytol kinase activity . If it is desired to down-regulate the expression of a given gene, i.e., decrease the expression of a gene through any means, such as by about 25%, 50%, 75% or more at the mRNA or protein level, a nucleic acid molecule comprising (i.e., in the

case of an RNA vector) or encoding (i.e., in the case of a DNA vector) an antisense nucleic acid molecule (see, e.g., Smith et al., Nature 334: 724-726 (1988)) to an RNA molecule transcribed from an aforementioned gene, for example, a dsRNAi molecule (see, e.g., Waterhouse et al., PNAS USA 95: 13959-13964 (1998)), a nucleic acid molecule, the expression of which results in the sense suppression (see, e.g., Napoli et al., Plant Cell 2: 279-289 (1989); U.S. Patent Nos. 5,190,931; 5,107,065; and 5,283,323; and international application publication no. WO 01/14538) of a gene encoding an LTT1 polypeptide or a nucleotide sequence encoding a phytol kinase polypeptide or a polypeptide having phytol kinase activity, or a nucleic acid molecule comprising a ribozyme to an RNA molecule transcribed from such a gene (see, for example, Senior, Biotech. Genet. Eng. Rev. 15: 79-119 (1998); Bird et al., Biotech. Genet. Eng. Rev. 9: 207-227(1991); Matzke et al., Trends Genet. 11(1): 1-3 (1995); Baulcombe, Plant Mol. Biol. 32(1-2): 79-88 (1996); Castanatto et al., Crit. Rev. Eukaryot. Gene Exp. 2(4): 331-357 (1992); Rossi, Trends Biotechnol. 13(8): 301-306 (1995); and WO 97/10328) can be utilized. Other techniques include promoter silencing (see, e.g., Park et al., Plant J. 9(2): 183-194 (1996)) and the use of DNA binding proteins (Beerli et al., PNAS USA 95: 14628-14633 (1997); and Liu et al., PNAS USA 94: 5525-5530 (1998)).

In antisense technology, the nucleic acid sequence generally is substantially identical to at least a portion, such as at least about 100 (or 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 750, 1,000, 1,500, 2,000 or more, up to the full-length of the gene, which is defined as a particular sequence of nucleotides along a molecule of DNA, which represents a functional unit of inheritance) contiguous nucleotides, of the endogenous gene or gene to be repressed, but need not be identical. The introduced sequence also need not be full-length relative to either of the primary transcription product or the fully processed mRNA. Generally, higher homology can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homologous non-coding segments can be equally effective.

If desired, antisense nucleic acid molecules can be chemically synthesized or enzymatically ligated using procedures known in the art. For example, an antisense nucleic acid can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides.

Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation and operably linked to a promoter. Preferably, production of antisense nucleic acids in plants occurs by means of a stably integrated transgene comprising a promoter operative in plants, an antisense oligonucleotide, and a terminator. The gene can be polycistronic, i.e., can comprise sequences from more than one gene, and can include sequences that correspond to a 5' UTR, a 3' UTR, an intron, and combinations thereof.

The plant cell, plant tissue, plant organ or plant is then contacted with the antisense nucleic acid molecules or with a construct encoding an antisense nucleic acid molecule such that the anti-sense strand of RNA is produced *in vivo*. In plant cells, it has been shown that anti-sense RNA inhibits gene expression (see, e.g., Sheehy et al., *PNAS USA* 85: 8805-8809 (1988); and U.S. Pat. Nos. 4,801,340 and 5,107,065). The antisense molecules can bind to genomic DNA or cellular mRNA so as to inhibit transcription or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex or by binding to DNA duplexes through specific interactions in the major groove of the double helix. Antisense nucleic acid molecules can be modified to target selected cells, i.e., via linking to a peptide or antibody (or antigenically reactive fragment thereof) that binds to a cell-surface molecule or receptor, and then administered systemically. Inhibition of expression of a given gene can be confirmed in a transformed plant cell by standard methods for measuring the presence and/or activity of a given protein. In this regard, it is important to point out that some plants contain two genes, i.e., "paralogs," encoding a given polypeptide. In such instances, a single antisense RNA molecule can be used to reduce and even block the expression of both paralogs, if so desired, depending on the antisense molecule utilized. However, in some instances, it may be desirable to down-regulate one paralog, but not the other.

dsRNA-dependent post-transcriptional gene silencing or RNAi is now used extensively in various diploid organisms. dsRNA-induced silencing phenomena are present in evolutionarily diverse organisms, including plants (see, e.g., U.S. Pat. No. 6,506,559; U.S. Pat. App. Pub. No. 2002/0168707; and int'l pat. app. pub. nos. WO 99/53050 and WO 99/61631), fungi, and metazoans (Hammond et al., Nat. Rev. Genet. 2: 110-119 (2001)). Stable silencing has been induced in model organisms by directed expression of long dsRNAs (Kennerdell et al., Nat. Biotechnol. 18: 896-898 (2000); Smith et al., Nature (London) 407: 319-320 (2000); and Tavernarakis et al., Nat. Genet. 24: 180-183 (2000)).

dsRNAi constructs can comprise as few as 21 nucleotides in sense and antisense orientation, or as many as 50, 75, 100, 125, 150, 175, 200 or more nucleotides in sense and antisense orientation.

Another method of down-regulating an LTT1 gene or nucleotide sequences encoding phytol kinase polypeptides and polypeptides having phytol kinase activity is sense supression. Sense suppression is the reduction in expression levels, usually at the level of RNA, of a particular endogenous gene or gene family by the expression of a homologous sense construct that is capable of transcribing mRNA of the same strandedness as the transcript of the endogenous gene (Napoli et al., Plant Cell 2: 279-289 (1990); van der Krol et al., Plant Cell 2: 291-299 (1990)). Suppression can result from stable transformation with a single copy nucleic acid molecule that is homologous to a nucleic acid sequence found within the cell (Prolls et al., Plant J. 2: 465-475 (1992)) or with multiple copies of a nucleic acid molecule that is homologous to a nucleic acid sequence found within the cell (Mittlesten et al., Mol. Gen. Genet. 244: 325-330 (1994)). Genes, even though different, linked to homologous promoters can result in suppression of the linked genes (Vaucheret, C. R. Acad. Sci. III 316a: 1471-1483 (1993); Flavell, PNAS USA 91: 3490-3496 (1994); van Blokland et al., Plant J. 6: 861-877 (1994); Jorgensen, Trends Biotechnol. 8: 340-344 (1990); Meins et al., In: *Gene Activation and Homologous Recombination in Plants*, Paszkowski, ed., pp. 335-348, Kluwer Academic, Netherlands (1994); Kinney, Induced Mutations and Molecular Techniques for Crop Improvement, Proceedings of a Symposium (19-23 June 1995; jointly organized by IAEA and FA), pp. 101-113 (IAEA-SM 340-49); and Que et al., *Dev. Genet.* 22(1): 100-109 (1998), and Smyth, *Curr. Biol.* 7(12): R793-R795 (1997). In sense technology, the nucleic acid sequence generally is substantially identical to at least a portion, such as at least about 21(or 50, 75, 100, 125, 150, 175, 200 or more) contiguous nucleotides, of the endogenous gene or gene to be repressed, but need not be identical.

Still yet another method is the use of a dominant negative mutant. For example, a dominant negative mutant of a polypeptide having phytol kinase activity as described herein can be generated by completely or partially deleting the C-terminal coding sequence, in particular all or part of the C-terminal coding sequence that is highly conserved among the polypeptides described herein. The resulting mutant can be operably linked to a promoter, such as an embryo-specific promoter from maize, for example, and cloned into a vector for introduction into a corn plant or part thereof. See, e.g., Jasinski et al., Plant Physiol. 130: 1871-1882 (2002)).

Ribozymes also have been reported to have use as a means to inhibit expression of endogenous plant genes (see, e.g., Merlo et al., Plant Cell 10(10): 1603-1622 (1998)). It is possible to design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered and is, thus, capable of recycling and cleaving other molecules, making it a true enzyme. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs. The design and use of target RNA-specific ribozymes is described in Haseloff et al., *Nature* 334: 585-591 (1988). Preferably, the ribozyme comprises at least about 20 continuous nucleotides complementary to the target sequence on each side of the active site of the ribozyme.

Alternatively, reverse genetics systems, which are well-known in the art, can be used to generate and isolate down-regulated or null mutants. One such system, the Trait Utility System for Corn, i.e., TUSC, is based on successful systems from other organisms (Ballinger et al., PNAS USA 86: 9402-9406 (1989); Kaiser et al., PNAS USA 87: 1686-1690 (1990); and Rushforth et al., Mol. Cell. Biol. 13: 902-910 (1993)). The central feature of the system is to identify *Mu* transposon insertions within a DNA sequence of interest in anticipation that at least some of these insertion alleles will be mutants. To develop the system in corn, DNA was collected from a large population of Mutator transposon stocks that were then self-pollinated to produce F2 seed. To find *Mu* transposon insertions within a specified DNA sequence, the collection of DNA samples is screened via PCR using a gene-specific primer and a primer that anneals to the inverted repeats of *Mu* transposons. A PCR product is expected only when the template DNA comes from a plant that contains a *Mu* transposon insertion within the target gene. Once such a DNA sample is identified, F2 seed from the corresponding plant is screened for a transposon insertion allele. Transposon insertion mutations of the *an1* gene have been obtained via the TUSC procedure (Bensen et al., Plant Cell 7: 75-84 (1995)). This system is applicable to other plant species, at times modified as necessary in accordance with knowledge and skill in the art.

T-DNA insertional mutagenesis can be used to generate insertional mutations in one of the above-mentioned genes so as to affect adversely the expression of a given gene. T-DNA tagged lines of plants can be screened using PCR. For example, a primer can be designed for one end of the T-DNA and another primer can be designed for the gene of interest and both primers can be used in PCR. If no PCR product is obtained, then there is no

insertion in the gene of interest. In contrast, if a PCR product is obtained, then there is an insertion in the gene of interest. Insertional mutations, however, often generate null alleles, which can be lethal. Alternatively, if there is more than one gene that encodes for a given enzyme, a mutation in one of the genes may not result in decreased expression of the enzyme encoded by the gene.

Another alternative method to decrease expression of a given gene is to use a compound that inhibits expression of one of the above-mentioned genes or that inhibits the activity of the protein encoded by one of the above-mentioned genes. In this regard, x-ray or gamma radiation can be used as can chemical mutagens, such as ethyl methyl sulfonate (EMS) or dimethyl butyric acid (DMB).

In addition to the above, gene replacement technology can be used to increase or decrease expression of a given gene. Gene replacement technology is based upon homologous recombination (see, Schnable et al., Curr. Opinions Plant Biol. 1: 123 (1998)). The nucleic acid of the enzyme of interest can be manipulated by mutagenesis (e.g., insertions, deletions, duplications or replacements) to either increase or decrease enzymatic function. The altered sequence can be introduced into the genome to replace the existing, e.g., wild-type, gene via homologous recombination (Puchta and Hohn, Trends Plant Sci. 1: 340 (1996); Kempin et al., Nature 389: 802 (1997)).

Down regulating phytol kinase polypeptides and polypeptides having phytol kinase activity prevents the plant from recycling free phytol from chlorophyll degradation for tocopherol biosynthesis. Therefore, a seed with high tocotrienol content (preferably >75%) can be obtained by seed specific expression of tyrA, HPPD, and seed specific antisense or antisense with a constitutive promoter of LTT1. Additional seed-specific expression of other tocopherol genes such as HPT, TMT2, GMT, and tocopherol cyclase that express proteins with preference for tocotrienol precursors as substrates may even further enhance tocotrienol biosynthesis. Such enzymes may be found in monocotyledone plants such as oil palm, rice, corn, wheat and other monocotyledone plants that naturally accumulate large amounts of tocotrienols.

In a preferred aspect, a similar genetic background is a background where the organisms being compared share about 50% or greater of their nuclear genetic material. In a more preferred aspect a similar genetic background is a background where the organisms being compared share about 75% or greater, even more preferably about 90% or greater of their nuclear genetic material. In another even more preferable aspect, a similar genetic

background is a background where the organisms being compared are plants, and the plants are isogenic except for any genetic material originally introduced using plant transformation techniques.

Exogenous genetic material may be transferred into a host cell by the use of a DNA vector or construct designed for such a purpose. Design of such a vector is generally within the skill of the art (*See, Plant Molecular Biology: A Laboratory Manual*, Clark (ed.), Springer, New York, 1997).

A construct or vector may include a plant promoter to express an mRNA that is translated into the polypeptide of choice. In a preferred embodiment, any nucleic acid molecules described herein can be operably linked to a promoter region which functions in a plant cell to cause the production of an mRNA molecule. For example, any promoter that functions in a plant cell to cause the production of an mRNA molecule, such as those promoters described herein, without limitation, can be used. In a preferred embodiment, the promoter is a plant promoter or a plant virus promoter.

A number of promoters that are active in plant cells have been described in the literature. These include the 7alpha' promoter, the USP88 promoter (U.S. Patent Application 10/429,516, filed May 5, 2003), the nopaline synthase (NOS) promoter (Ebert *et al., Proc. Natl. Acad. Sci. (U.S.A.)*, 84:5745-5749, 1987), the octopine synthase (OCS) promoter (which is carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*). Examples of constitutive promoters that are active in plant cells include, but are not limited to the nopaline synthase (P-NOS) promoters; the cauliflower mosaic virus (P-CaMV) 19S and 35S (P-CaMV35S, U.S. Patent 5,858,642) and enhanced versions of the CaMV 35S promoter (P-CaMV35S-enh, U.S. Patent 5,322,938; the figwort mosaic virus promoter (P-FMV35S, U.S. Patents 6,051,753 and 6,018,100); and actin promoters, such as the rice actin promoter (P-Os.Act1, U.S. Patent 5,641,876), the Adh promoter (Walker *et al., Proc. Natl. Acad. Sci. (U.S.A.)*, 84:6624-6628, 1987), the sucrose synthase promoter (Yang *et al., Proc. Natl. Acad. Sci. (U.S.A.)*, 87:4144-4148, 1990), the R gene complex promoter (Chandler *et al., The Plant Cell*, 1:1175-1183, 1989) and the chlorophyll a/b binding protein gene promoter, *etc.* These promoters have been used to create DNA constructs that have been expressed in plants. Promoters known or found to cause transcription of DNA in plant cells can be used in the invention. The sequences of the promoters disclosed in these referenced patents are herein incorporated by reference.

For the purpose of expression in source tissues of the plant, such as the leaf, seed, root or stem, it is preferred that the promoters utilized have relatively high expression in these specific tissues. Tissue-specific expression of a protein of the present invention is a particularly preferred embodiment. For this purpose, one may choose from a number of promoters for genes with tissue- or cell-specific or enhanced expression. Examples of such promoters reported in the literature include the chloroplast glutamine synthetase GS2 promoter from pea (Edwards *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 87:3459-3463, 1990), the chloroplast fructose-1,6-biphosphatase (FBPase) promoter from wheat (Lloyd *et al.*, *Mol. Gen. Genet.*, 225:209-216, 1991), the nuclear photosynthetic ST-LS1 promoter from potato (Stockhaus *et al.*, *EMBO J.*, 8:2445-2451, 1989), the serine/threonine kinase (PAL) promoter and the glucoamylase (CHS) promoter from *Arabidopsis thaliana*. Also reported to be active in photosynthetically active tissues are the ribulose-1,5-bisphosphate carboxylase (RbcS) promoter from eastern larch (*Larix laricina*), the promoter for the *cab* gene, cab6, from pine (Yamamoto *et al.*, *Plant Cell Physiol.*, 35:773-778, 1994), the promoter for the Cab-1 gene from wheat (Fejes *et al.*, *Plant Mol. Biol.*, 15:921-932, 1990), the promoter for the CAB-1 gene from spinach (Lubberstedt *et al.*, *Plant Physiol.*, 104:997-1006, 1994), the promoter for the cab1R gene from rice (Luan *et al.*, *Plant Cell.*, 4:971-981, 1992), the pyruvate, orthophosphate dikinase (PPDK) promoter from corn (Matsuoka *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 90:9586-9590, 1993), the promoter for the tobacco Lhcb1*2 gene (Cerdan *et al.*, *Plant Mol. Biol.*, 33:245-255, 1997), the *Arabidopsis thaliana* SUC2 sucrose-H+ symporter promoter (Truernit *et al.*, *Planta.*, 196:564-570, 1995) and the promoter for the thylakoid membrane proteins from spinach (*psa*D, *psa*F, *psa*E, PC, FNR, *atp*C, *atp*D, *cab*, *rbc*S). Other promoters for the chlorophyll a/b-binding proteins may also be utilized in the invention, such as the promoters for *Lhc*B gene and *Psb*P gene from white mustard (*Sinapis alba*; Kretsch *et al.*, *Plant Mol. Biol.*, 28:219-229, 1995).

For the purpose of expression in sink tissues of the plant, such as the tuber of the potato plant, the fruit of tomato, or the seed of corn, wheat, rice and barley, it is preferred that the promoters utilized in the invention have relatively high expression in these specific tissues. A number of promoters for genes with tuber-specific or tuber-enhanced expression are known, including the class I patatin promoter (Bevan *et al.*, *EMBO J.*, 8:1899-1906, 1986); Jefferson *et al.*, *Plant Mol. Biol.*, 14:995-1006, 1990), the promoter for the potato tuber ADPGPP genes, both the large and small subunits, the sucrose synthase promoter (Salanoubat and Belliard, *Gene*, 60:47-56, 1987), Salanoubat and Belliard, *Gene*, 84:181-185,

1989), the promoter for the major tuber proteins including the 22 kd protein complexes and protease inhibitors (Hannapel, *Plant Physiol.*, 101:703-704, 1993), the promoter for the granule-bound starch synthase gene (GBSS) (Visser *et al.*, *Plant Mol. Biol.*, 17:691-699, 1991) and other class I and II patatins promoters (Koster-Topfer *et al.*, *Mol. Gen. Genet.*, 219:390-396, 1989); Mignery *et al.*, *Gene*, 62:27-44, 1988).

Other promoters can also be used to express a polypeptide in specific tissues, such as seeds or fruits. Indeed, in a preferred embodiment, the promoter used is a seed specific promoter. Examples of such promoters include the 5' regulatory regions from such genes as napin (Kridl *et al., Seed Sci. Res.* 1:209:219, 1991), phaseolin (Bustos, *et al., Plant Cell,* 1(9):839-853, 1989), soybean trypsin inhibitor (Riggs, *et al., Plant Cell,* 1(6):609-621, 1989), ACP (Baerson, *et al., Plant Mol. Biol.,* 22(2):255-267, 1993), stearoyl-ACP desaturase (Slocombe, *et al., Plant Physiol.,* 104(4):167-176, 1994), soybean a' subunit of β-conglycinin (Chen *et al., Proc. Natl. Acad. Sci.,* 83:8560-8564, 1986), and oleosin (see, for example, Hong, *et al., Plant Mol. Biol.,* 34(3):549-555, 1997). Further examples include the promoter for β-conglycinin (Chen *et al., Dev. Genet.,* 10:112-122, 1989). Also included are the zeins, which are a group of storage proteins, found in corn endosperm. Genomic clones for zein genes have been isolated (Pedersen *et al.*, Cell 29:1015-1026, 1982), and Russell *et al.*, *Transgenic Res.*, 6(2):157-168, 1997) and the promoters from these clones, including the 15 kD, 16 kD, 19 kD, 22 kD, 27 kD and genes, could also be used. Other promoters known to function, for example, in corn include the promoters for the following genes: *waxy, Brittle, Shrunken 2*, Branching enzymes I and II, starch synthases, debranching enzymes, oleosins, glutelins and sucrose synthases. A particularly preferred promoter for corn endosperm expression is the promoter for the glutelin gene from rice, more particularly the Osgt-1 promoter (Zheng *et al.*, *Mol. Cell Biol.*, 13:5829-5842, 1993). Examples of promoters suitable for expression in wheat include those promoters for the ADPglucose pyrosynthase (ADPGPP) subunits, the granule bound and other starch synthase, the branching and debranching enzymes, the embryogenesis-abundant proteins, the gliadins and the glutenins. Examples of such promoters in rice include those promoters for the ADPGPP subunits, the granule bound and other starch synthase, the branching enzymes, the debranching enzymes, sucrose synthases and the glutelins. A particularly preferred promoter is the promoter for rice glutelin, Osgt-1. Examples of such promoters for barley include those for the ADPGPP subunits, the granule bound and other starch synthase, the branching enzymes, the

debranching enzymes, sucrose synthases, the hordeins, the embryo globulins and the aleurone specific proteins.

The seed-specific promoters that include the 5' regulatory regions of the napin gene provide expression of transgenes in seed tissues (U.S. Patents 5,420,034 and 6,459,018, herein incorporated by reference). In soybean, 7S refers to β-conglycinin, a major class of seed storage proteins. The trimeric β-conglycinin is comprised of the α, α′ and β subunits. Expression of 7Sα′ has been well studied by many researchers over the years. The 7Sα′ subunit is expressed at mid to late stages of seed development. A transgene encoding the α′-subunit of soybean β-conglycinin showed seed-specific expression in petunia (Beachy *et al.*, EMBO J. 4:3047-3053, 1985). Functional analysis of the regulatory elements indicated that a 900 bp upstream fragment of the 7Sα′ promoter contains the necessary elements to produce seed-specific expression in transgenic petunia (Chen *et al.*, Proc. Natl. Acad. Sci. 83:8560-8564, 1986). The ovule-specific promoter for BEL1 gene can also be used (Reiser *et al.* Cell 83:735-742, 1995, GenBank No. U39944; Ray *et al,* Proc. Natl. Acad. Sci. *U.S.A.* 91:5761-5765, 1994). The egg and central cell specific MEA (FIS1) and FIS2 promoters are also useful reproductive tissue-specific promoters (Luo *et al., Proc. Natl. Acad. Sci. (U.S.A.),* 97:10637-10642, 2000; Vielle-Calzada, *et al.*, Genes Dev. 13:2971-2982, 1999). Additional promoters useful for driving expression of a transgene in seed tissues are described in numerous references, for example, U.S. Patents 6,437,220; 6,426,447; 6,342,6571 6,410,828; 5,767,363 and 5,623,067, herein incorporated by reference)

A preferred promoter for expression in the seed is a napin promoter. Another preferred promoter for expression is an Arcelin5 promoter (U.S. Patent Publication 2003/0046727). Additional promoters that may be utilized are described, for example, in U.S. Patents 5,378,619; 5,391,725; 5,428,147; 5,447,858; 5,608,144; 5,608,144; 5,614,399; 5,633,441; 5,633,435; and 4,633,436.

Constructs or vectors may also include, with the coding region of interest, a nucleic acid sequence that acts, in whole or in part, to terminate transcription of that region. A number of such sequences have been isolated, including the Tr7 3' sequence and the NOS 3' sequence (Ingelbrecht *et al., The Plant Cell,* 1:671-680, 1989); Bevan *et al., Nucleic Acids Res.*, 11:369-385, 1983). Regulatory transcript termination regions can be provided in plant expression constructs of this invention as well. Transcript termination regions can be provided by the DNA sequence encoding the gene of interest or a convenient transcription termination region derived from a different gene source, for example, the transcript

termination region that is naturally associated with the transcript initiation region. The skilled artisan will recognize that any convenient transcript termination region that is capable of terminating transcription in a plant cell can be employed in the constructs of the present invention, *e.g.*, TML 3' from *Agrobacterium tumefaciens* Ti plasmid.

A vector or construct may also include regulatory elements. Examples of such include the Adh intron 1 (Callis *et al.*, *Genes and Develop.*, 1:1183-1200, 1987), the sucrose synthase intron (Vasil *et al.*, *Plant Physiol.*, 91:1575-1579, 1989) and the TMV omega element (Gallie *et al.*, *The Plant Cell*, 1:301-311, 1989). These and other regulatory elements may be included when appropriate.

A vector or construct may also include a selectable marker. Selectable markers may also be used to select for plants or plant cells that contain the exogenous genetic material. Examples of such include, but are not limited to: a *neo* gene (Potrykus *et al.*, *Mol. Gen. Genet.*, 199:183-188, 1985), which codes for kanamycin resistance and can be selected for using kanamycin, RptII, G418, hpt *etc.*; a bar gene which codes for bialaphos resistance; a mutant EPSP synthase gene (Hinchee *et al.*, *Bio/Technology,* 6:915-922, 1988); Reynaerts *et al.,* Selectable and Screenable Markers. In Gelvin and Schilperoort. Plant Molecular Biology Manual, Kluwer, Dordrecht (1988); Reynaerts *et al.,* Selectable and screenable markers. In Gelvin and Schilperoort. Plant Molecular Biology Manual, Kluwer, Dordrecht (1988), and (Jones *et al., Mol. Gen. Genet.*, 1987), which encodes glyphosate resistance; a nitrilase gene which confers resistance to bromoxynil (Stalker *et al.*, *J. Biol. Chem.* 263:6310-6314, 1988); a mutant acetolactate synthase gene (ALS) which confers imidazolinone or sulphonylurea resistance (European Patent Application 154,204 (Sept. 11, 1985), ALS (D'Halluin *et al., Bio/Technology*, 10:309-314, 1992), and a methotrexate resistant DHFR gene (Thillet *et al.*, *J. Biol. Chem.* 263:12500-12508, 1988).

A vector or construct may also include a screenable marker. Screenable markers may be used to monitor expression. Exemplary screenable markers include: a β-glucuronidase or *uid*A gene (GUS) which encodes an enzyme for which various chromogenic substrates are known (Jefferson, *Plant Mol. Biol, Rep.*, 5:387-405, 1987); Jefferson *et al., EMBO J.*, 6:3901-3907, 1987); an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.*, Stadler Symposium, 11:263-282, 1988); a β-lactamase gene (Sutcliffe *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 75:3737-3741, 1978), a gene which encodes an enzyme for which various chromogenic substrates are known (*e.g.*, PADAC, a chromogenic cephalosporin); a luciferase gene (Ow *et*

al., *Science*, 234:856-859, 1986); a *xyl*E gene (Zukowsky *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 80:1101-1105, 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an α-amylase gene (Ikatu *et al.*, *Bio/Technol.*, 8:241-242, 1990); a tyrosinase gene (Katz *et al.*, *J. Gen. Microbiol.*, 129:2703-2714, 1983) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to melanin; an α-galactosidase, which will turn a chromogenic α-galactose substrate.

Included within the terms "selectable or screenable marker genes" are also genes that encode a secretable marker whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers that encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes that can be detected catalytically. Secretable proteins fall into a number of classes, including small, diffusible proteins that are detectable, (*e.g.*, by ELISA), small active enzymes that are detectable in extracellular solution (*e.g.*, α-amylase, β-lactamase, phosphinothricin transferase), or proteins that are inserted or trapped in the cell wall (such as proteins that include a leader sequence such as that found in the expression unit of extension or tobacco PR-S). Other possible selectable and/or screenable marker genes will be apparent to those of skill in the art.

In a preferred embodiment of the invention, a transgenic plant expressing the desired protein is to be produced. Various methods for the introduction of a desired polynucleotide sequence encoding the desired protein into plant cells are available and known to those of skill in the art and include, but are not limited to: (1) physical methods such as microinjection, electroporation, and microprojectile mediated delivery (biolistics or gene gun technology); (2) virus mediated delivery methods; and (3) *Agrobacterium*-mediated transformation methods.

The most commonly used methods for transformation of plant cells are the *Agrobacterium*-mediated DNA transfer process and the biolistics or microprojectile bombardment mediated process (*i.e.*, the gene gun). Typically, nuclear transformation is desired but where it is desirable to specifically transform plastids, such as chloroplasts or amyloplasts, plant plastids may be transformed utilizing a microprojectile-mediated delivery of the desired polynucleotide.

Agrobacterium-mediated transformation is achieved through the use of a genetically engineered soil bacterium belonging to the genus *Agrobacterium*. A number of wild type and disarmed strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* harboring Ti or Ri plasmids can be used for gene transfer into plants. Gene transfer is done via the transfer

of a specific DNA known as "T-DNA" that can be genetically engineered to carry any desired piece of DNA into many plant species.

Agrobacterium-mediated genetic transformation of plants involves several steps. The first step, in which the virulent *Agrobacterium* and plant cells are first brought into contact with each other, is generally called "inoculation". Following the inoculation, the *Agrobacterium* and plant cells/tissues are permitted to be grown together for a period of several hours to several days or more under conditions suitable for growth and T-DNA transfer. This step is termed "co-culture". Following co-culture and T-DNA delivery, the plant cells are treated with bactericidal or bacteriostatic agents to kill the *Agrobacterium* remaining in contact with the explant and/or in the vessel containing the explant. If this is done in the absence of any selective agents to promote preferential growth of transgenic versus non-transgenic plant cells, then this is typically referred to as the "delay" step. If done in the presence of selective pressure favoring transgenic plant cells, then it is referred to as a "selection" step. When a "delay" is used, one or more "selection" steps typically follow it.

With respect to microprojectile bombardment (U.S. Patents 5,550,318; 5,538,880; and 5,610,042; each of which is specifically incorporated herein by reference in its entirety), particles are coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, platinum, and preferably, gold.

An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is the Biolistics Particle Delivery System (BioRad, Hercules, CA), which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with monocot plant cells cultured in suspension.

Microprojectile bombardment techniques are widely applicable, and may be used to transform virtually any plant species. Examples of species that have been transformed by microprojectile bombardment include monocot species such as maize (PCT Publication WO 95/06128), barley, wheat (U.S. Patent 5,563,055, incorporated herein by reference in its entirety), rice, oat, rye, sugarcane, and sorghum; as well as a number of dicots including tobacco, soybean (U.S. Patent 5,322,783, incorporated herein by reference in its entirety), sunflower, peanut, cotton, tomato, and legumes in general (U.S. Patent 5,563,055, incorporated herein by reference in its entirety).

To select or score for transformed plant cells regardless of transformation methodology, the DNA introduced into the cell contains a gene that functions in a regenerable plant tissue to produce a compound that confers upon the plant tissue resistance

to an otherwise toxic compound. Genes of interest for use as a selectable, screenable, or scorable marker would include but are not limited to GUS, green fluorescent protein (GFP), luciferase (LUX), antibiotic or herbicide tolerance genes. Examples of antibiotic resistance genes include the penicillins, kanamycin (and neomycin, G418, bleomycin); methotrexate (and trimethoprim); chloramphenicol; kanamycin and tetracycline.

The regeneration, development, and cultivation of plants from various transformed explants are well documented in the art. This regeneration and growth process typically includes the steps of selecting transformed cells and culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil. Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports regeneration of plants. Developing plantlets are transferred to soil less plant growth mix, and hardened off, prior to transfer to a greenhouse or growth chamber for maturation.

The present invention can be used with any transformable cell or tissue. By transformable as used herein is meant a cell or tissue that is capable of further propagation to give rise to a plant. Those of skill in the art recognize that a number of plant cells or tissues are transformable in which after insertion of exogenous DNA and appropriate culture conditions the plant cells or tissues can form into a differentiated plant. Tissue suitable for these purposes can include but is not limited to immature embryos, scutellar tissue, suspension cell cultures, immature inflorescence, shoot meristem, nodal explants, callus tissue, hypocotyl tissue, cotyledons, roots, and leaves.

Any suitable plant culture medium can be used. Examples of suitable media would include but are not limited to MS-based media (Murashige and Skoog, Physiol. Plant, 15:473-497, 1962) or N6-based media (Chu *et al.*, *Scientia Sinica* 18:659, 1975) supplemented with additional plant growth regulators including but not limited to auxins, cytokinins, ABA, and gibberellins. Those of skill in the art are familiar with the variety of tissue culture media, which when supplemented appropriately, support plant tissue growth and development and are suitable for plant transformation and regeneration. These tissue culture media can either be purchased as a commercial preparation, or custom prepared and modified. Those of skill in the art are aware that media and media supplements such as nutrients and growth regulators for use in transformation and regeneration and other culture

conditions such as light intensity during incubation, pH, and incubation temperatures that can be optimized for the particular variety of interest.

Any of the nucleic acid molecules of the invention may be introduced into a plant cell in a permanent or transient manner in combination with other genetic elements such as vectors, promoters, enhancers, *etc.* Further, any of the nucleic acid molecules of the invention may be introduced into a plant cell in a manner that allows for expression or overexpression of the protein or fragment thereof encoded by the nucleic acid molecule.

The present invention also provides for parts of the plants, particularly reproductive or storage parts, of the present invention. Plant parts, without limitation, include seed, endosperm, ovule and pollen. In a particularly preferred embodiment of the present invention, the plant part is a seed. In one embodiment the seed (or grain) is a constituent of animal feed.

In another embodiment, the plant part is a fruit, more preferably a fruit with enhanced shelf life. In another preferred embodiment, the fruit has increased levels of a tocopherol. In another preferred embodiment, the fruit has increased levels of a tocotrienol.

Any of the plants or parts thereof of the present invention may be processed to produce a feed, meal, protein, or oil preparation, including oil preparations high in total tocopherol content and oil preparations high in any one or more of each tocopherol component listed herein. A particularly preferred plant part for this purpose is a seed. In a preferred embodiment the feed, meal, protein or oil preparation is designed for livestock animals or humans, or both. Methods to produce feed, meal, protein and oil preparations are known in the art. See, for example, U.S. Patents 4,957,748; 5,100,679; 5,219,596; 5,936,069; 6,005,076; 6,146,669; and 6,156,227. In a preferred embodiment, the protein preparation is a high protein preparation. Such a high protein preparation preferably has a protein content of greater than about 5% w/v, more preferably 10% w/v, and even more preferably 15% w/v. In a preferred oil preparation, the oil preparation is a high oil preparation with an oil content derived from a plant or part thereof of the present invention of greater than 5% w/v, more preferably 10% w/v, and even more preferably 15% w/v. In a preferred embodiment the oil preparation is a liquid and of a volume greater than about 1, 5, 10 or 50 liters. The present invention provides for oil produced from plants of the present invention or generated by a method of the present invention. Such an oil may exhibit enhanced oxidative stability. Also, such oil may be a minor or major component of any resultant product. Moreover, such oil may be blended with other oils. In a preferred embodiment, the oil produced from plants of

the present invention or generated by a method of the present invention constitutes greater than about 0.5%, 1%, 5%, 10%, 25%, 50%, 75% or 90% by volume or weight of the oil component of any product. In another embodiment, the oil preparation may be blended and can constitute greater than about 10%, 25%, 35%, 50% or 75% of the blend by volume. Oil produced from a plant of the present invention can be admixed with one or more organic solvents or petroleum distillates.

Descriptions of breeding methods that are commonly used for different traits and crops can be found in one of several reference books (*e.g.*, Hayward, *Plant Breeding: Principles and Prospects*, Vol 1, Chapman & Hall; ISBN: 0412433907 (1993); Richards, A.J., *Plant Breeding Systems,* Stanley Thornes Pub Ltd; 2nd ed., ISBN: 0412574500 (1997); Allard, R.W., *Principles of Plant Breeding,* 2nd ed., John Wiley & Sons, ISBN: 0471023094 (1999)

A transgenic plant of the present invention may also be reproduced using apomixis. Apomixis is a genetically controlled method of reproduction in plants where the embryo is formed without union of an egg and a sperm. Apomixis is economically important, especially in transgenic plants, because it causes any genotype, no matter how heterozygous, to breed true. Thus, with apomictic reproduction, heterozygous transgenic plants can maintain their genetic fidelity throughout repeated life cycles. Methods for the production of apomictic plants are known in the art, *e.g.*, U.S. Patent 5,811,636.

OTHER ORGANISMS

A nucleic acid of the present invention may be introduced into any cell or organism such as a mammalian cell, mammal, fish cell, fish, bird cell, bird, algae cell, algae, fungal cell, fungi, or bacterial cell. A protein of the present invention may be produced in an appropriate cell or organism. Preferred host and transformants include: fungal cells such as *Aspergillus*, yeasts, mammals, particularly bovine and porcine, insects, bacteria, and algae. Particularly preferred bacteria are *Agrobacteruim tumefaciens* and *E. coli.*

Methods to transform such cells or organisms are known in the art (EP 0 238 023; Yelton *et al., Proc. Natl. Acad. Sci. (U.S.A.)*, 81:1470-1474, 1984); Malardier *et al., Gene*, 78:147-156, 1989); Becker and Guarente, In: Abelson and Simon (eds.), *Guide to Yeast Genetics and Molecular Biology, Method Enzymol.*, 194:182-187, Academic Press, Inc., New York; Ito *et al., J. Bacteriology*, 153:163, 1983) Hinnen *et al., Proc. Natl. Acad. Sci. (U.S.A.)*, 75:1920, 1978); Bennett and LaSure (eds.), *More Gene Manipulations in fungi,*

Academic Press, CA (1991). Methods to produce proteins of the present invention are also known (Kudla *et al.*, *EMBO*, 9:1355-1364, 1990); Jarai and Buxton, *Current Genetics*, 26:2238-2244 (1994); Verdier, *Yeast*, 6:271-297, 1990; MacKenzie *et al.*, *Journal of Gen. Microbiol.*, 139:2295-2307, 1993); Hartl *et al.*, *TIBS*, 19:20-25, 1994); Bergenron *et al.*, *TIBS*, 19:124-128, 1994); Demolder *et al.*, *J. Biotechnology*, 32:179-189, 1994); Craig, *Science*, 260:1902-1903, 1993); Gething and Sambrook, *Nature*, 355:33-45, 1992); Puig and Gilbert, *J. Biol. Chem.*, 269:7764-7771, 1994); Wang and Tsou, *FASEB Journal*, 7:1515-1517, 1993); Robinson *et al.*, *Bio/Technology*, 1:381-384, 1994); Enderlin and Ogrydziak, *Yeast*, 10:67-79, 1994); Fuller *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 86:1434-1438, 1989); Julius *et al.*, *Cell*, 37:1075-1089, 1984); Julius *et al.*, *Cell*, 32:839-852, 1983).

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, or 99% identity to such sequences, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, or 99% identity to such sequences, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of α-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, or 99% identity to such sequences, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of γ-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, or 99% identity to such sequences, provides in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of β-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, or 99% identity to such sequences, provides in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of δ-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, or 99% identity to such

sequences, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, or 99% identity to such sequences, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of α-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, or 99% identity to such sequences, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of γ-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, or 99% identity to such sequences, provides in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of δ-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising SEQ ID NOs: 1, 5, 17, or sequences having at least about 70, 80, 90, 95, or 99% identity to such sequences, provides in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of β-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or polypeptide having phytol kinase activity provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or a polypeptide having phytol kinase activity, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of α-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or a polypeptide having phytol kinase activity, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of γ-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or a polypeptide having phytol

kinase activity, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of β-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or a polypeptide having phytol kinase activity, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of δ-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide, or a polypeptide having phytol kinase activity, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide, or a polypeptide having phytol kinase activity, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of α-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide, or a polypeptide having phytol kinase activity, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of γ-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or a polypeptide having phytol kinase activity, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of β-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a phytol kinase polypeptide or a polypeptide having phytol kinase activity, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of δ-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of α-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of γ-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of β-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of δ-tocopherols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of α-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed cell,

relative to an untransformed cell with a similar genetic background, an increased level of γ-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of β-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20-41 and 53-68, provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of δ-tocotrienols.

In a preferred embodiment, DNA constructs of the present invention comprising nucleic acid molecules encoding polypeptides of the present invention provide in a transformed cell, relative to an untransformed cell with a similar genetic background, an increased level of plastoquinols.

Any of a variety of methods may be used to obtain one or more of the above-described nucleic acid molecules (Zamechik *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 83:4143-4146, 1986); Goodchild *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 85:5507-5511, 1988); Wickstrom *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 85:1028-1032, 1988); Holt *et al.*, *Molec. Cell. Biol.*, 8:963-973, 1988); Gerwirtz *et al.*, *Science*, 242:1303-1306, 1988); Anfossi *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 86:3379-3383, 1989); Becker *et al.*, *EMBO J.*, 8:3685-3691, 1989). Automated nucleic acid synthesizers may be employed for this purpose. In lieu of such synthesis, the disclosed nucleic acid molecules may be used to define a pair of primers that can be used with the polymerase chain reaction (Mullis *et al.*, *Cold Spring Harbor Symp. Quant. Biol.*, 51:263-273, 1986); Erlich *et al.*, European Patent 50,424; European Patent 84,796; European Patent 258,017; European Patent 237,362; Mullis, European Patent 201,184; Mullis *et al.*, U.S. Patent 4,683,202; Erlich, U.S. Patent 4,582,788; and Saiki *et al.*, U.S. Patent 4,683,194) to amplify and obtain any desired nucleic acid molecule or fragment.

Promoter sequences and other genetic elements, including but not limited to transcriptional regulatory flanking sequences, associated with one or more of the disclosed nucleic acid sequences can also be obtained using the disclosed nucleic acid sequence provided herein. In one embodiment, such sequences are obtained by incubating nucleic acid

molecules of the present invention with members of genomic libraries and recovering clones that hybridize to such nucleic acid molecules thereof. In a second embodiment, methods of "chromosome walking," or inverse PCR may be used to obtain such sequences (Frohman *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 85:8998-9002, 1988); Ohara *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)*, 86:5673-5677, 1989); Pang *et al.*, *Biotechniques*, 22:1046-1048, 1977); Huang *et al.*, *Methods Mol. Biol.*, 69:89-96, 1997); Huang *et al.*, *Method Mol. Biol.*, 67:287-294, 1997); Benkel *et al.*, *Genet. Anal.*, 13:123-127, 1996); Hartl *et al.*, *Methods Mol. Biol.*, 58:293-301, 1996). The term "chromosome walking" means a process of extending a genetic map by successive hybridization steps.

Another subset of the nucleic acid molecules of the invention includes nucleic acid molecules that are markers. The markers can be used in a number of conventional ways in the field of molecular genetics. Such markers include nucleic acid molecules homologous or complementary to SEQ ID NOs: 1, 5, or 17 and fragments thereof that can act as markers and other nucleic acid molecules of the present invention that can act as markers.

It is understood that one or more of the nucleic acid molecules of the invention may be used as molecular markers. It is also understood that one or more of the protein molecules of the invention may be used as molecular markers.

In an aspect of the present invention, one or more of the nucleic molecules of the present invention are used to determine the level of expression (*i.e.*, the concentration of mRNA in a sample, *etc.*) in a plant (preferably canola, corn, *Brassica campestris,* oilseed rape, rapeseed, soybean, crambe, mustard, castor bean, peanut, sesame, cottonseed, linseed, safflower, oil palm, flax or sunflower) or pattern (*i.e.*, the kinetics of expression, rate of decomposition, stability profile, *etc.*) of the expression of a protein encoded in part or whole by one or more of the nucleic acid molecule of the present invention

A number of methods can be used to compare the expression between two or more samples of cells or tissue. These methods include hybridization assays, such as northerns, RNAse protection assays, and *in situ* hybridization. Alternatively, the methods include PCR-type assays. In a preferred method, the expression response is compared by hybridizing nucleic acids from the two or more samples to an array of nucleic acids. The array contains a plurality of suspected sequences known or suspected of being present in the cells or tissue of the samples.

Having now generally described the invention, the same will be more readily understood through reference to the following examples that are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE 1

This example sets forth the identification and characterization of the *Arabidopsis thaliana* LTT1 mutant. Mutagenized (M_2) seeds of *Arabidopsis thaliana,* ecotypes Columbia and Lansberg were obtained both by purchase from Lehle Seeds (Round Rock, TX, U.S.A.) and by standard ethane methyl sulfonate (EMS) (a.k.a. Ethyl methanesulfonate, Sigma-Aldrich, St. Louis, MO, U.S.A.) mutagenesis methodology. The M_2 plants were grown from the M_2 seeds in greenhouse conditions with one plant per 2.5 inch pot. The resulting M_3 seeds were collected from individual M_2 plants and analyzed for tocopherol levels.

Approximately 10,000 M_3 seeds of *Arabidopsis thaliana*, ecotypes Landsberg and Columbia, were analyzed for individual tocopherol levels using the following procedure. Five milligrams of seeds from individual plants were ground to a fine powder and then extracted with 200 microliter (µl) of a 1% pyrogallol (Sigma-Aldrich, St. Louis, MO, U.S.A.) in ethanol solution. This mixture was allowed to incubate at 4°C for 60 minutes prior to filtering (Whatman UniFilter® plate, PVDF 0.45 µm, Whatman, Scarborough, ME, U.S.A.). The filtrate was then analyzed for tocopherol content and composition by fractionating the mixture using a Waters model 2790 high performance liquid chromatography (HPLC) system (Waters Corporation, Milford, MA, U.S.A.) equipped with a 4.6 x 250 mm Zorbax silica reversed phase column (Agilent Technologies, U.S.A.). Tocopherol and metabolites were detected using a Waters model 474 fluorescence detector with excitation set at 290 nanometer (nm), emittance at 336 nm, and bandpass and slits set at 30 nm. The elution program used an isocratic flow of 10% methyl-tert-butyl-ether (MTBE) (Sigma-Aldrich, St. Louis, MO, U.S.A.) in hexane at a rate of 1.5 milliliter (ml)/minute for 12 minutes. Prior to each injection, a clean up run of 75% MTBE in hexane was performed for 3 minutes, followed by a re-equilibration step of 10% MTBE in hexane for 3 minutes.

Individual plant lines with total tocopherol levels lower than wild type were reanalyzed in the next generation (M4) to confirm their heritability. One *Arabidopsis* LTT mutant line was identified and designated LTT1. The LTT1 mutant line produced 127 ng total tocopherol/mg seed versus 438 nanogram (ng) total tocopherol/milligram (mg) seed

observed in the non-mutagenized *Arabidopsis* wild type control. This equates to about a 75% reduction in total seed tocopherol levels.

EXAMPLE 2

This example sets forth the identification and sequencing of the mutant LTT1 gene in the *Arabidopsis thaliana* low total tocopherol mutants. The mutant LTT1 gene was mapped between markers T32M21_29601 and T32M21_66646 on chromosome V. This region contains seven open reading frames. This entire 37 kilobase (kb) region was sequenced, using polymerase chain reaction (PCR) techniques well known in the art, from the LTT1 mutant line described in EXAMPLE 1 and compared to the wild type nucleic acid sequence for this region. Analysis of this region in the LTT1 mutant line revealed that one of the open reading frames, T32M21_90 (SEQ ID NO: 3) contained a point mutation resulting in the conversion of the amino acid tryptophan to a stop codon at amino acid position 227, relative to the ATG (SEQ ID NO: 4). The corresponding wild type polynucleic acid sequence for LTT1 was SEQ ID NO: 1 which encodes the LTT1 polypeptide SEQ ID NO: 2.

EXAMPLE 3

This example describes the identification of the LTT1-r gene from *Arabidopsis thaliana*. The LTT1-r polypeptide sequence (SEQ ID NO: 6) (NCBI General Identifier gi: 15237702) was identified in the NCBI database by a BLAST[blastp] and BLAST[PSI] alignment searches (NCBI) using the LTT1 polypeptide sequence (SEQ ID NO: 2) (gi: 15238184) as the query sequence. Like the LTT1 polypeptide (SEQ ID NO: 2), the LTT1-r polypeptide (SEQ ID NO: 6) also contains six transmembrane domains and a chloroplast target peptide and shares 38% identity with LTT1.

EXAMPLE 4

This example sets forth the transformation and expression of a wild type *Arabidopsis* LTT1 gene in *Arabidopsis thialiana*. The LTT1 (SEQ ID NO: 1) full-length cDNA was excised from an EST clone, CPR208415, with SalI and BamHI restriction enzymes and operably linked to the napin promoter and napin 3' termination sequences at SalI and BglII restriction sites in sense orientation with respect to the napin promoter in pMON36525 (Figure 2) to generate a recombinant binary vector pMON69914 (Figure 3). The sequence of the LTT1 (SEQ ID NO: 1) cDNA was confirmed by sequencing with napin 5'-sense (SEQ ID NO: 7) and napin 3'-antisense (SEQ ID NO: 8) nucleic acid primers using standard sequencing methodology.

The plant binary vector pMON69914 (Figure 3) was used in *Arabidopsis thaliana* plant transformation to direct the expression of the LLT1 (SEQ ID NO: 1) in the embryo. The binary vector was transformed into ABI strain *Agrobacterium* cells by electroporation (Bio-Rad electroprotocol manual, Dower *et al., Nucleic Acids Res.* 16:6127-6145, 1988). Transgenic *Arabidopsis thaliana* plants were obtained by *Agrobacterium*-mediated transformation as described by Valverkens *et al., Proc. Nat. Acad. Sci. 85*:5536-5540,1988), Bent *et al., Science,* 265:1856-1860, 1994), and Bechtold *et al., C.R. Acad. Sci., Life Sciences* 316:1194-1199, 1993). Transgenic plants were selected by sprinkling the transformed T_1 seeds onto the selection plates containing MS basal salts (4.3 g/L), Gamborg'a B-5, 500X (2.0 g/L), sucrose (10 g/L), MES (0.5 g/L), phytagar (8 g/L), carbenicillin (250 mg/L), cefotaxime (100 mg/L), plant preservation medium (2 ml/L), and kanamycin (60 mg/L) and then vernalizing them at 4°C in the absence of light for 2-4 days. The seeds were transferred to 23°C, and 16/8 hours light/dark cycle for 5-10 days until seedlings emerge. After one set of true leaves were formed on the kanamycin resistant seedlings, they were transferred to soil and grown to maturity. The T_2 seed harvested from the transformants was analyzed for tocopherol content. The plant binary vector pMON69914 (Figure 3) was also transformed into the LTT1 mutant lines of *Arabidopsis thaliana* by the same plant transformation method described above.

EXAMPLE 5

This example sets forth the results of expressing a wild type *Arabidopsis* LTT1 gene (SEQ ID NO: 1) in wild type or LTT1 mutant *Arabidopsis* plants. A binary vector pMON69914 (Figure 3) carrying a P-napin::*Arabidopsis LTT1*::napin 3' expression cassette was transformed into wild type Columbia *Arabidopsis* and LTT1 mutant *Arabidopsis* lines as described in Example 4, and seeds from the transgenic *Arabidopsis* lines were analyzed for seed total tocopherol levels. As shown in Table 2, the over expression of *Arabidopsis* LTT1 (SEQ ID NO: 1) in transgenic wild type *Arabidopsis* increases seed total tocopherol levels in all lines tested. In one case (Col-0 LTT1-1), the tocopherol level was significantly greater than the empty vector control as determined using the Tukey-Kramer HSD statistical test set at a 95% confidence level (alpha=0.05) (JMP statistical software, SAS Institute, Cary, NC, U.S.A.). Wild type empty vector control seed produced a mean total tocopherol level of 448.3 ng/mg seed. The transgenic *Arabidopsis* LTT1 mutant lines carrying the pnapin::*Arabidopsis LTT1*::napin 3' expression cassette produced mean seed total tocopherol levels that ranged from 454.0 to 477.0 ng/mg.

Table 2. Total seed tocopherol levels in T3 *Arabidopsis* seed lines expressing the LTT1 (SEQ ID NO: 1) gene.

Line	N	Mean Total Seed Tocopherol level (ng/mg)	Std Error		
Col-0 LTT1-1	12	477.0	5.4	A	
Col-0 LTT1-2	10	453.0	5.9		B
Col-0 LTT1-3	12	461.1	5.4	A	B
Col-0 LTT1-4	12	469.0	5.4	A	B
Col-0 LTT1-5	8	454.0	6.6	A	B
Empty vector control	7	448.3	7.0		B

Lines not designated by same letter (either A or B) are significantly different from one another.

Comparisons for all pairwise combinations using Tukey-Kramer HSD, Alpha=0.05

Over expression of the LTT1 gene (SEQ ID NO: 1) in the LTT1 mutant line restored wild type levels of seed tocopherols (Table 3). Both the LTT1 mutant line and the LTT1 mutant line transformed with an empty vector control produced tocopherol levels of approximately 90 ng/mg. When expressed in the mutant LTT1 background, the functional wild type LTT1 lines produced seed total tocopherol levels of approximately 365 ng/mg. The tocopherol levels observed in the wild type LTT1 (SEQ ID NO: 1) line was significantly greater than that observed in both the empty vector and LTT1 mutant lines as determined using the Tukey-Kramer HSD test set at a 95% confidence level (alpha=0.05) (JMP statistical software, SAS Institute, Cary, NC, U.S.A.).

Table 3. Total seed tocopherol levels in mutant LTT1 *Arabidopsis* seed lines expressing the LTT1 (SEQ ID NO: 1) gene

Line	N	Mean Total Seed Tocopherol level (ng/mg)	Std Dev		
LTT1 (SEQ ID NO: 1)	20	364.5	38.7	A	
Empty vector control	4	86.6	1.2		B
LTT1 Mutant	2	91.2	6.5		B

Lines not designated by same letter (either A or B) are significantly different from one another.

Comparisons for all pairs using Tukey-Kramer HSD, Alpha=0.05.

EXAMPLE 6

This example sets forth the transformation and expression of a wild type *Arabidopsis* LTT1 gene in soybean plants to increase total seed tocopherol levels. To direct the expression of *Arabidopsis* LTT1 gene (SEQ ID NO: 1) in soybean seed, a binary construct with LTT1 operably linked to a seed-specific USP88 (seed protein from *Vicia faba*) promoter and operably linked to a 3' TML termination sequence is prepared (pMON81063) (Figure 8).

Other soybean seed-specific promoters such as 7Sα, 7Sα' and arcelin-5 can also be used. Other termination sequences such as pea rubisco small subunit 3'(T-Ps.RbcS) and arcelin 3' can also be used. Vector construction for the LTT1 construct is performed using standard cloning techniques well established in the art and described in lab manuals such as Sambrook *et al.*, 2001. The control vector (pMON69969) (Figure 9) contains a T-DNA with a selectable marker cassette. Finally, an assortment of transformation strategies, such as co-transformation and re-transformation, all well known in the art, can be employed to direct these genes in an assortment of combinations into the soybean plant.

Transgenic soybean seeds generated with the LTT1 constructs are analyzed for total seed tocopherol and tocotrienol levels as described in Example 1. Total seed tocopherol and tocotrienol levels are significantly higher in the LTT1 (SEQ ID NO: 1) transformed plant lines than those of the empty vector control lines as determined using statistical tests such as the Tukey-Kramer HSD test set at a 95% confidence level (alpha=0.05) (JMP statistical software, SAS Institute, Cary, NC, U.S.A.).

EXAMPLE 7

This example sets forth the transformation and expression of a wild type *Arabidopsis* LTT1 gene (SEQ ID NO: 1) in combination with other tocopherol pathway genes in soybean plants to increase total seed tocopherol levels. To demonstrate the *in planta* performance of the LTT1 nucleic acid sequence with other tocopherol pathway genes, a soybean binary vector (pMON77670) (Figure 4) containing the LTT1 gene (SEQ ID NO: 1) driven by a USP promoter and a 3' TML termination sequence is prepared to direct the expression of LTT1 in soybean seeds, an *Arabidopsis* geranylgeranyl hydrogenase (GGH_{At}) (SEQ ID NO: 13), an *Arabidopsis* homogentisate phytyltransferase (HPTAt) (SEQ ID NO: 15), an *Arabidopsis* *p*-hydroxyphenylpyruvate dioxygenase ($HPPD_{At}$)(SEQ ID NO: 14) and an *Erwinia herbicola* prephenate dehydrogenase ($tyrA_{Eh}$)(SEQ ID NO: 16). The specific nucleic acid sequences selected and used herein are examples only. Other GGH, HPT, HPPD and TyrA sequences are known and can be used. The Synechocystis LTT1 (SEQ ID NO: 17) or other nucleic acids (N-terminally fused to CTP, if needed) of the present invention could be substituted for SEQ ID NO: 1.

Construction of the 5-gene vector (pMON77670)(Figure 4), as well as the control vector (pMON77637), is performed using standard cloning techniques well established in the art (Sambrook *et al.*, 2001). The LTT1 gene construct (pMON81019) (Figure 5) is digested with Bsp120I and NotI restriction enzymes and the resulting nucleic acid fragment is inserted

into the NotI site of the 4-gene vector (pMON77637) (Figure 6) containing expression cassettes for a 7Salpha promoter::(GGHAt)::E9 3 '-termination sequence, an arcelin-5 promoter::(HPTAt)::arcelin-3'sequence, a 7Salpha ' promoter::CTP1::HPPDAt::E9-3 ' termination sequence, and a 7Salpha ' promoter::CTP2::TyrAEh::E9-3 ' termination sequence. The 4-gene vector pMON77637 serves as the control vector for measuring the effects of LTT1 on seed total tocopherol levels.

Tocopherol pathway genes that are useful for optimal tocopherol biosynthesis, such as GGH, HPPD, tyrA, GGPPS, HPT, DXS, DXR GMT, TMT2, and LTT1 can be prepared by codon optimization to optimally express in soybean or any other commercially important transgenic crop to further boost the tocopherol production in oil seeds. For codon opimization references, see, *e.g.,* GenBank, National Center Biotechnology Information, USA; see U.S. Patent 5,689,052), and FindPatterns (Genetics Computer Group, Inc., USA), which is a database of 20 known 5-6 nucleotide long motifs that are known to be associated with mRNA instability (*i.e.,* premature polyadenylation signals.

Finally, an assortment of transformation strategies, such as co-transformation and re-transformation, all well known in the art, can be employed to direct these genes in an assortment of combinations into the soybean plant.

Total seed tocopherol and tocotrienol levels are significantly higher in plant lines transformed with the aforementioned tocopherol pathway genes than those of the LTT1 minus control lines as determined using statistical tests such as the Tukey-Kramer HSD test set at a 95% confidence level (alpha=0.05) (JMP statistical software, SAS Institute, Cary, NC, U.S.A.).

EXAMPLE 8

This example sets forth the identification and characterization of a *Synechocystis* LTT1 homolog. A BLASTP search (National Center for Biotechnology Information, NIH, U.S.A.) of a *Synechocystis* PCC6803 genomic nucleic acid sequence database using the *Arabidopsis* LTT1 nucleotide sequence (SEQ ID: 1) as the query sequence identified a nucleic acid sequence , slr1652 (SEQ ID NO: 17), as an *Arabidopsis* LTT1 homolog (E value of $5X10^{-11}$, with 29% identity over a 237 residue stretch). A *Synechocystis* mutant cyanobacteria colony was created by inactivating the slr1652 gene to show that slr1652 functions in tocopherol synthesis and accumulation. Four nucleic acid PCR primer pairs, designated SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, were designed based on the *Synechocystis* genomic nucleic acid sequence including and flanking

the slr1652 gene. Using standard PCR amplification protocols (Sambrook *et al.*, 2001) and *Synechocystis* PCC6803 genomic DNA and SEQ ID NO: 9 and SEQ ID NO: 10, and separately, SEQ ID NO: 11 and SEQ ID NO: 12, nucleic acid primer sequences, two PCR products of approximately 0.45 kb, corresponding to sequences surrounding the upstream and downstream regions of the slr1652 nucleic acid sequence, were produced. The PCR products were successively cloned as NotI/BamHI and BamHI/XhoI restriction fragments into a pBluescriptII KS(+) plasmid (Stratagene, CA, U.S.A.), to recreate an approximately 0.9 kb region encompassing the slr1652 gene, with a unique BamHI site marking the deletion of approximately 236 bp within the slr1652 coding region. A 1.25 kb kanamycin resistance cassette from a pKISS plasmid (Pharmacia Corporation, St. Louis, MO, U.S.A.) was cloned as an Ecl136II restriction fragment into the slr1652 BamHI site after blunting with T4 DNA polymerase. Plasmid pMON78621 (Figure 7) with the kanamycin cassette oriented in the same direction as the internally truncated slr1652 gene was obtained and confirmed by nucleic acid sequencing.

Chromosomal knock-out mutants of the slr1652 gene were obtained by the transformation of pMON78621 into *Synechocystis* PCC6803 and selection of transconjugants on medium supplemented with 5 mg/L kanamycin, as described (Williams, *Methods Enzymology,* 167:766-778 (1988). The medium for the growth of cells was BG-11 (Sigma-Aldrich Inc., St. Louis, MO, U.S.A.), supplemented with 5 mM TES (N-[Tris(hyrdoxymethyl)methyl]-2-aminoethanesulfonic acid) (Sigma-Aldrich, St. Louis, MO, U.S.A.) pH 8.0. Kanamycin-resistant transconjugants were sub-cultured by re-streaking on kanamycin containing medium 4-5 times, and two single colony isolates were established and designated as strains 1652-KO-1 and 1652-KO-2. When partially purified genomic DNA from these two strains, as well as the wild type parent were used as templates for PCR using the primers SEQ ID NO: 9 and SEQ ID NO: 12, an amplified product of ~1.1 kb was produced from the wild type DNA. Strains 1652-KO-1 and 1652-KO-2 yielded a product of ~2.1 kb and none of the ~1.1 kb product. PCR analysis clearly showed that the slr1652 genomic region had been faithfully deleted by homologous recombination in both mutants, and wild type copies of the gene were no longer present. The growth rate of both mutants was not significantly different from the wild type parent, showing that slr1652 function is not essential for *Synechocystis* growth and development.

Liquid cultures of wild type *Synechocystis* PCC6803 and both mutants were grown under light in BG-11 medium + 5 mM TES (+ 5 mg/lit kanamycin for the mutants) with

shaking at 30°C to a final density of ~2.0-2.5 as measured by absorbance at 730 nm (an absorbance of 1.0 correspond to a cell density of ~$4X10^8$ cells/mL). Cells corresponding to 10.0 A_{730} units were harvested, extracted and analyzed for their tocopherol content as described in EXAMPLE 1. The wild type and 1652-KO-1 and 1652-KO-2 strains had a total tocopherol content of 80.5, 42.0 and 21.0 ng/A_{730} units, respectively (n=2). The 50-75% reduction of total tocopherol in the slr1652 knock-out mutants is similar to the phenotype seen in the *Arabidopsis* LTT1 mutants described in EXAMPLE 2 demonstrating that LTT1 and slr1652 are homologs that perform the same function in plants and cyanobacteria.

EXAMPLE 9

This example sets forth the transformation and expression of an LTT1 (SEQ ID NO: 1) or LTT1-r (SEQ ID NO: 5) gene in combination with a *Synechocystis* chlorophyllase gene (SEQ ID NO:18 or SEQ ID NO:19) to increase total seed tocopherol levels in *Synechosystis.*

Pfam analysis (Pfam version 9.0 (May 2003), Washington University, St. Louis, MO, USA; Pfam is a large collection of multiple sequence alignments and hidden Markov models covering many common protein families) of LTT1 revealed that LTT1 and LTT1-r are members of the DUF56 gene family of putative integral membrane proteins. While the function of the DUF56 family is unknown, members of the family include a dolichol kinase (EC:2.7.1.108) termed Sec59, and a phosphatidate cytidylyltransferase (EC:2.7.7.41), termed CDS, also known as CDP-diacylglycerol synthase, both isolated from yeast.

CDS is the enzyme that catalyzes the synthesis of CDP-diacylglycerol from CTP and phosphatidate (PA). CDS is a membrane-bound enzyme, and contains the N-terminal consensus sequence S-x-[LIVMF]-K-R-x (4)-K-D-x- [GSA]-x (2)-[LIF]-[PG]-x-H-G-G-[LIVMF]-x-D-R- [LIVMFT]-D (SeqLab®, GCG Wisconsin Package, 2001-2003 Accelrys Inc.). LTT1 and LTT1-r are not cytidyltransferases since they lack the CDS consensus sequence.

Based on multiple sequence alignments as well as an examination of key structural and phylogenic motifs, *e.g.*, substrate and consensus recognition sequences, LTT1 and LTT1-r are not functional homologs of Sec59, rhodopsin or CDS in *Arabidopsis.* LTT1 is a novel enzyme of the DUF56 gene family. The likely substrate for LTT1 and LTT1-r is phytol, a molecule that is structurally similar to retinol and dolichol. The likely primary function of LTT1 is to phosphorylate phytol. This function is consistent with the low tocopherol phenotype observed in the *Arabidopsis* and *Synechocystis* LTT1 mutant lines

based on a model in which a portion of the phytoldiphosphate used for tocopherol biosynthesis is provided by phytol liberated from metabolized chlorophyll rather than directly from geranylgeranyldiphosphate reduction to phytoldiphosphate. This position is supported by the fact that there is an inverse relationship between chlorophyll degradation and tocopherol synthesis in, for example, canola; as the concentration of chlorophyll goes down the concentration of tocopherol increases.

To increase phytoldiphosphate availability in a seed, the LTT1 gene (SEQ ID NO: 1) from *A. thaliana* or its homolog from *Synechocystis* (SEQ ID NO: 17) (which is operably linked 3'to a chloroplast target peptide such as the *Arabidopsis* ribulose bisphosphate carboxylase small subunit (CTP1)), are operably linked to a seed-specific promoter, such as the 7S alpha promoter or the napin promoter, which is in turn operably linked to a 3' sequence such as the Nos 3' sequence, the E9 3' sequence, or the napin 3' sequence. This expression cassette is combined with a seed-specific expression cassette for a chlorophyllase, such as the *Arabidopsis* chlorophyllase 1 (gi:30912637) (SEQ ID NO: 18), or the *Arabidopsis* chlorophyllase 2 (gi:30912739, gi:6729677) (SEQ ID NO: 19). The chlorophyllase is expressed using the 7S alpha' promoter, the USP88 promoter, or the napin promoter and an appropriate 3' sequence such as the TML 3' sequence, or the E9 3' sequence. These two expression cassettes are transformed into a plant binary vector (*e.g.*, a soy binary vector, see Figure 3) and further transformed via *Agrobacterium* mediated transformation into soybean. Transgenic seed are analyzed for changes in tocopherol content and composition as described in Example 1.

Total seed tocopherol and tocotrienol levels are significantly higher in plant lines transformed with the aforementioned tocopherol pathway genes than those of the empty vector control lines as determined using statistical tests such as the Tukey-Kramer HSD test set at a 95% confidence level (alpha=0.05) (JMP statistical software, SAS Institute, Cary, NC, U.S.A.).

EXAMPLE 10

This example describes an assay which can be used to determine the amount of phytol in tissue extracts. This method determines phytol levels in liquid extracts of various biological materials, such as plants, by use of gas chromatography coupled with a time of flight mass spectrometer. The quantification was accomplished using an external standard curve constructed by using a unique mass that represents phytol and using the retention time of the phytol eluting from the gas chromatograph. The identity of the phytol was further

confirmed by comparing the mass spectra at this retention time with the reference standard. The detection limit of the instrument was 0.05 nanograms per microliter. The method detection limit depends on the extract solution and the amount of noise created by the extract sample itself. Thus, method detection limits were determined on a sample-by-sample basis. The analysis time was less than 4 minutes per sample.

The gas chromatograph was an Agilent 6890 chromatograph (Agilent Technologies, U.S.A.). The gas chromatograph column was a DB5. The dimensions of the column were 10 meters in length, with an internal diameter of 180 microns, and a film thickness of 0.18. The carrier gas was helium and flowed through the column at a rate of 1.5 mL/minute. A constant flow was maintained throughout the programmed temperature ramp of the chromatograph. The initial temperature of the column was 130°C with no hold time. The temperature was increased at a rate of 30°C per minute until the column reaches 270°C and was held at 270°C for 2 minutes. One microliter of extract was injected into the injection port of the gas chromatograph. The mode of injection was splitless. The temperature of the injector was 250°C.

The mass of the phytol molecules was determined using a Pegasus® III (LECO Corporation, U.S.A.) time of flight mass spectrometer (TOFMS). The outlet of the Agilent gas chromatograph column was placed through a heated transfer line (250°C) into the TOFMS. The instrument was operated in electron impact mode with ionzation energy of 70 electron volts. The source was operated at a temperature of 200°C and the detector voltage was 1600 volts. The mass to charge range was set to be between 45 and 305 units.

The standard curve was constructed using commercial standards (Sigma-Aldrich, St. Louis, MO, U.S.A.). The standard curve was an external curve and ranged in concentration from 7 nanograms per microliter to 0.14 nanograms per microliter. The R squared value for the curve was 0.9993. The mass to charge used for quantification was 71. The retention time was 178 seconds for the cis isomer and 183 seconds for the trans isomer.

EXAMPLE 11

This example illustrates the increase in phytol levels resulting from inactivation of the *Arabidopsis thaliana* LTT1 (SEQ ID NO: 1) gene or the *Synechocystis* LTT1 (SEQ ID NO: 17) gene. As shown in Figure 1, Geranylgeranioldiphosphate can serve as a substrate for chlorophyll synthase to form geranylated chlorophyll (Grassl *et al., Planta* 213:620-628, 2001). Geranylated chlorophyll is then reduced to phytylated chlorophyll in a reaction catalyzed by geranylgeranioldiphosphate reductase (chlP). Phytylated chlorophyll accounts

for the majority of chlorophyll in plants. When phytylated chlorophyll is degraded by chlorophyllase, free phytol is released. As chlorophyll degradation increases post anthesis, the level of phytol increases and the phytol is activated by phytol kinase (LTT1) to produce phytyl pyrophosphate (Phytyl-PP), which is a substrate for HPT. The increase in Phytyl-PP substrate leads to an increase in tocopherol production. If the activity of LTT1 is blocked, the result is an increase in cell phytol levels. This effect is illustrated for *Arabidopsis thaliana* LTT1 mutant seed and *Synechocystis* LTT1 mutant cells in Table 4. These results show that seed phytol levels increase from 0.203 ng/mg to 0.595 ng/mg when *Arabidopsis thaliana* LTT1 (SEQ ID NO: 1) is inactivated. Similarly, when *Synechocystis* LTT1 (SEQ ID NO: 17) is inactivated, cell phytol levels increase from undetectable levels to 0.482 ng/mg.

Table 4. Phytol levels in wild type and mutant *Arabidopsis thaliana* seeds and *Synechocystis* cells

Sample ID	N	Phytol Content	Std. Dev.
A thaliana wild type seed	2	101.7	9.1
A thaliana LTT1 mutant seed	2	297.4	34.2
Synechocystis wild type cells	2	Not detectable	Not detectable
Synechocystis LTT1 mutant cells	4	241.0	17.1

The phytol content in *Arabidopsis thaliana* is expressed as ng/mg.
The phytol content in *Synechocystis* cells is expressed as ng per OD unit at 730 nm.

EXAMPLE 12.

This example sets forth the transformation and expression of a wild type *Arabidopsis* LTT1 gene in combination with a chlorophyllase gene and other tocopherol pathway genes in soybean to increase total seed tocopherol levels. As illustrated in EXAMPLE 11, chlorophyll degradation by the chlorophyllase can increase seed phytol levels. When used in combination with LTT1 (SEQ ID NO: 1) and other tocopherol pathway genes, total seed tocopherol levels are increased. To demonstrate the *in planta* performance of the LTT1 nucleic acid sequence in combination with a chlorophyllase gene and other tocopherol pathway genes, a soybean binary vector (pMON77670) (Figure 4) containing the LTT1 gene (SEQ ID NO: 1) driven by a USP promoter and a 3' TML termination sequence is prepared to direct the expression of LTT1 in soybean seeds in combination with an *Arabidopsis* chlorophyllase (SEQ ID NO: 18 or SEQ ID NO: 19), an *Arabidopsis* geranylgeranyl hydrogenase (GGH_{At}) (SEQ ID NO: 13), an *Arabidopsis* homogentisate phytyltransferase (HPTAt) (SEQ ID NO: 15), an *Arabidopsis p*-hydroxyphenylpyruvate dioxygenase ($HPPD_{At}$)(SEQ ID NO: 14) and an *Erwinia herbicola* prephenate dehydrogenase

(*tyrA*$_{Eh}$)(SEQ ID NO: 16). The specific nucleic acid sequences selected and used herein are examples only. Other GGH, HPT, HPPD and tyrA sequences are known and can be used. Similarly, other chlorophyllases can be used, preferably those with native CTPs. The Synechocystis LTT1 (SEQ ID NO: 17) or other nucleic acids (N-terminally fused to CTP, if needed) of the present invention could be substituted for SEQ ID NO: 1.

Construction of the 5-gene vector (pMON77670)(Figure 4), as well as the control vector (pMON77637), is performed using standard cloning techniques well established in the art (Sambrook *et al.*, 2001). The LTT1 gene construct (pMON81019) (Figure 5) is digested with Bsp120I and NotI restriction enzymes and the resulting nucleic acid fragment is inserted into the NotI site of the 4-gene vector (pMON77637) (Figure 6) containing expression cassettes for a 7Salpha promoter::(GGHAt)::E9 3 '-termination sequence, an arcelin-5 promoter::(HPTAt)::arcelin-3'sequence, a 7Salpha ' promoter::CTP1::HPPDAt::E9-3 ' termination sequence, and a 7Salpha ' promoter::CTP2::TyrAEh::E9-3 ' termination sequence. A seed specific expression cassette for a plastid targeted chlorophyllase is added to pMON77670 using standard cloning techniques. The 4-gene vector pMON77637 serves as a control vector for measuring the effects of LTT1 on seed total tocopherol levels. Other controls include the 5-gene vector pMON77670 and the vector resulting from the combination of an expression cassette for a plastid targeted chlorophyllase with pMON77670.

As explained in Example 7, tocopherol pathway genes that are useful for optimal tocopherol expression, such as GGH, HPPD, tyrA, GGPPS, HPT, DXS, DXR GMT, TMT2, chlorophyllase, and LTT1 can be prepared by codon optimization to optimally express in soybean or any other commercially important transgenic crop to further boost the tocopherol production in oil seeds.

Co-transformation and re-transformation strategies are used to incorporate 4 to 8 or more tocopherol pathway genes to create transgenic lines expressing multiple tocopherol pathway genes.

Total seed tocopherol and tocotrienol levels are significantly higher in plant lines transformed with chlorophyllase, LTT1 (SEQ ID NO: 1) and the aforementioned tocopherol pathway genes when compared to control lines transformed with a similar vector lacking LTT1 and chlorophyllase as determined using statistical tests such as the Tukey-Kramer HSD test set at a 95% confidence level (alpha=0.05) (JMP statistical software, SAS Institute, Cary, NC, U.S.A.).

EXAMPLE 13

This example sets forth methods used to analyze LTT1 (SEQ ID NO: 2) and LTT1-r (SEQ ID NO: 6) amino acid sequences from a variety of biological sources in order to identify common structural motifs and sequence homologs contained therein. A variety of cDNA and genomic databases were searched and the data extracted from them analyzed using a suite of sequence search programs available from NCBI (National Center For Biotechnology Information, U.S.A.).

cDNA sequences from soybean (*Glycine max*), *Arabidopsis thaliana*, Corn (*Zea mays*), Leek (*Allium porrum*), wheat (*Triticum aestivum*), and rice (*Oryza sativa*) that were found to be homologous to *Arabidopsis thaliana* LTT1 were identified by searching EST (Expressed Sequence Tags) databases using the TBLASTN program (NCBI) and an E value criterion of $1e^{-5}$ or lower. The identities of these ESTs were confirmed by searching non-redundant databases using BLAST[blastx] (NCBI). ESTs with top blast hits to LTT1 or LTT1-r were extracted from the databases. Full insert sequences of the cDNA clones from the different EST sequences that aligned with the 5' region of LTT1 and LTT1-r were determined. The full insert sequence of the cDNA which covered the most 5' region of LTT1 and LTT1-r cDNAs was translated using in-house software and the encoded amino acid sequences were determined (SEQ ID NOs: 37-68).

Rice homologues (SEQ ID NOs: 46-52) of LTT1 (SEQ ID NO: 2) and LTT1-r ((SEQ ID NO: 6) were identified by searching an in-house rice genomic database using BLAST[blastp] (NCBI).

Cyanobacterial, Eubacterial and Archea amino acid sequences (SEQ ID NOs: 20-35, and 79) were obtained from GenBank® (NCBI). Yeast sequences sec59 (SEQ ID NO: 35) and Hsd1 (SEQ ID NO: 36) were also obtained from GenBank®. To show the relationship among these sequences (SEQ ID NOs: 2, 6, 20-35, 37-68, and 79) a phylogenetic tree was constructed (Figure 10). Sequences (representing SEQ ID NOs: 2, 6, 20-35, 37-68, and 79) were aligned with one another using the ClustalX multiple sequence alignment software (Jeanmougin *et al.*, *Trends Biochem. Sci.*, 23:403-405, 1998; Thompson *et al.*, *Nucleic Acids Research*, 24:4876-4882,1997). The multiple alignments of the protein sequences were visualized and edited using GeneDoc (Indiana University, IN, USA; Nicholas *et al.*, *Embnew.News*, 4:14, 1997). Portions of the sequences from yeast sec59 (SEQ ID NO: 35), yeast Hsd1(SEQ ID NO: 36) and BaProchloro2 (SEQ ID NO: 28), which introduced gaps in the amino acid sequence, were deleted from the multiple alignment. Portions of the N

terminal sequence, which did not align or were missing from several sequences, were also removed. The resulting optimized alignment was used to construct a phylogenetic tree using MEGA version 2.1 software (http://www.megasoftware.net/ (Kumar *et al.*, (2001) MEGA2: Molecular Evolutionary Genetics Analysis software, Arizona State University, AZ, USA.). The phylogenetic tree was refined using the gamma distance model with pair wise deletion. Bootstrapping was used to test the accuracy of the tree (1000 replications). The phylogenetic tree with the bootstrap values is shown in (Figure 10). All plant amino acid sequences split into two major groups (clades) exemplified by LTT1 (SEQ ID NO: 2) and LTT1-r (SEQ ID NO: 6). All the cyanobacterial sequences clustered together in a separate clade. Yeast sequences clustered separately in a yet another clade along with the Archea sequences.

From the multiple alignments, five plant phytol kinase motifs (SEQ ID NOs: 74-78) and five cyanobacterial phytol kinase motifs (SEQ ID NOs: 69-73) were used to identify plant and cyanobacterial phytol kinases. Plant motifs 1, 2, 3, 4 and 5 correspond to amino acids 101-122, 131-175, 187-122, 225 to 254 and 267-285 of Arabidopsis LTT1 (SEQ ID NO:2), respectively. Cyanobacterial motifs 1, 2, 3, 4 and 5 correspond to amino acids 43-66, 89-118, 129-144, 156-174, and 203-219, respectively of Synechocystis LTT1 homolog (SEQ ID NO: 79). The specificity of these motifs was tested using a Hidden Markov Model (HMM) that was built using an HMMER software package (Washington University, MO, USA; Eddy, *Bioinformatics,* 14:755-763, 1998). The non-redundant amino acid database from Genbank (NCBI), which contains more than 1.45 million protein sequences, was searched using HMM and the aforementioned motifs. Plant motifs 1, 4 and 5 (Figures 16, 19, and 20, SEQ ID NOs: 74, 77, and 78) are specific to plant phytol kinase sequences at an E value cut off of 1.0. Cyanobacterial motifs 3, 4, and 5 (SEQ ID NOs: 71, 72, and 73) are specific to Cyanobacterial sequences. Cyano motifs 4 and 5 are specific to cyanobacterial phytol kinases at an E value cut off of 1.0 and motif 3 is specific at an E value cutoff of 0.001.

EXAMPLE 14

This example sets forth a Phytol kinase assay. Phytol kinase activity is assayed according to a modified procedure of Inoue *et al., Phytochemistry* 40:377-381, 1995. The pH of the assay mixture is adjusted to pH 7.6, and tritiated phytol (Moravek Biochemicals, Inc., Brea, CA, U.S.A.) is used in place of farnesol. Ribonucleotide triphosphates such as CTP, ATP, GTP, or TTP are provided as phosphor donors. Additional cations such as Ca++ can be added as required. The enzyme reaction is terminated by the addition of a 2-fold volume of

chloroform/methanol (2:1). The assay mixture is centrifuged at 3000 x g for 15 min for phase separation. Aliquots of the aqueous and the organic layer are analyzed by HPLC. Samples of approximately 20 microliters are separated on a HP1100 series HPLC system (Hewlett-Packard, Agilent Technologies, U.S.A.) consisting of HP G1329A Auto Sampler, HP G1311A Quaternary Pump, HP G1315A Diode Array Detector, HP G1321A Fluorescence Detector, Packard Radiomatic 500TR Flow Scintillation Analyzer, connected to a 4.6 x 250 mm (5 µm) Vydac model 201TP54 C18 HPLC column (VYDAC, Hesperia, CA, U.S.A.). Phytol derivatives are monitored via the radiation emitted by the tritium label. The mobile phase used on the C18 column is a gradient consisting of two solvents. Solvent A is 25 mM $NaHCO_3$ in water, and solvent B is 100% acetonitrile (ACN). The gradient is initiated using a solvent mix of 70/30 A/B and increased to a solvent mix of 0/100 A/B over a 20-minute time period. From 20 to 39 minutes the gradient is maintained at 0/100 A/B. From 39 to 40 minutes the gradient ratio is shifted to 70/30 A/B. Retention times for metabolites are given in Table 5.

Table 5. Retention times (minutes) for metabolites fractionated be a C18 HPLC column

Metabolite	Retention Time (Minutes)
Gerranylgeranyl diphosphate	8.6
Phytyl diphosphate	10.2
Gerranylgeraniol	21.1
Phytol	26.4
Chlorophyll a	29.5
Chlorophyll b	31.5

EXAMPLE 15

This example sets forth the drought tolerance test that shows that *A. thaliana* plants transformed with a phytol kinase gene (LTT1; SEQ ID NO:1) are tolerant to drought relative to control plants that were not transformed with the LTT1 gene. The study design for this stress assay is a single factor design, with the LTT1 construct being the factor, where all experimental plants are exposed to a period of drought stress during flowering.

Seeds were stratified in 0.1% phytagar at 4°C in the dark for 3 days and then sown in flats filled with Metro-Mix® 200 (The Scotts® Company, U.S.A.). Humidity domes were then added to each flat and flats were assigned locations and placed in climate-controlled growth chambers. Plants were grown under a temperature regime of 22°C day and 20°C night, with a photoperiod of 16 hours and average light intensity of 170 µmol/m^2/s.

After the first true leaves appeared, humidity domes were removed and the plants were sprayed with BASTA™ herbicide in Silwet™ L-77 (OSI Specialties Inc., U.S.A.) at a mixture rate of 8.28 mL BASTA™ containing 18.2% active ingredient and 1 mL Silwet diluted to 20 L. After spraying, plants were put back in the growth chamber for 3 additional days. Flats were watered for 1 hour the week following the BASTA™ treatment. Watering was continued every seven days until the flower bud primordia became apparent (growth stage 5.10), at which time plants were watered for the last time. After the last watering, plants were covered with ARACON® (DuPont Company, U.S.A.) sleeves and placed on growth chamber drying racks.

Beginning ten days after the last watering, plants were examined daily until 4 plants/line had wilted. The proportions of wilted and non-wilted LTT1 transgenic and control plants were compared over each of the next six days and an overall log rank test was performed to compare the two survival curves using S-PLUS statistical software (S-PLUS 6, Guide to statistics, Insightful, Seattle, WA, U.S.A.). The results of that analysis (TABLE 6) show that the LTT1 (SEQ ID NO: 1) plants were significantly more tolerant to drought than the wild type control plants, which were not transformed with the LTT1 gene (p=0.0336). The mean number of days from last watering until wilting for the LTT1 transformed plants was 5.73 days and for the wild type controls was 4.71 days. At the end of the experiment, 86.4% of the LTT1 plants had wilted as compared to 100% of the wild type controls.

Table 6. Results of a log rank test for drought stress

line	Time To Wilting (days)	Mean days to wilting	p-value
LTT1-1	3,6,6,6,6+	5.73	0.034
LTT1-2	6,6,6,6+		
LTT1-3	6,6,6,6,6+		
LTT1-4	6,6,6,6		
LTT1-5	3,6,6,6		
Wild type control	3,3,3,6,6,6,6	4.71	

Log rank test (S-PLUS 6).

A "+" score signifies that the plant was not wilted at the conclusion of the test.

p-value is the probability that the difference in the LTT1 and Control survival curves is not due merely to chance.

Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and

detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.